THE CYTOLYTIC ACTION OF GUINEA PIG AND RABBIT COMPLEMENT ON SENSITIZED NUCLEATED MOUSE CELLS

a serological study of cell surface antigens

R.A.P. KOENE

PROMOTOR: PROF. DR. P. WIJDEVELD

CO-REFERENT: I.F.C. McKENZIE, M.D.

THE CYTOLYTIC ACTION OF GUINEA PIG AND RABBIT COMPLEMENT ON SENSITIZED NUCLEATED MOUSE CELLS

a serological study of cell surface antigens

ACADEMISCH PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE GENEESKUNDE
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN
OP GEZAG VAN DE RECTOR MAGNIFICUS
DR G. BRENNINKMEIJER, HOOGLERAAR
IN DE FACULTEIT DER SOCIALE WETENSCHAPPEN
VOLGENS HET BESLUIT VAN DE SENAAT
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 23 MAART 1972
DES NAMIDDAGS OM 4 UUR

DOOR

Robert Arnoud Pieter KOENE

GEBOREN TE MAASTRICHT

NIJMEGEN 1972

This study was made in collaboration with Ian F.C. McKenzie, M.D., in the laboratory of Henry J. Winn, Ph.D., Transplantation Unit (head: Paul S. Russell, M.D.), Massachusetts General Hospital, Boston, Mass., U.S.A.
Parts of it were presented at the Symposium on Immunogenetics of the H-2 System held in Prague on 1st-4th September 1970.
The study was supported by USPHS grants 761-8544-5 and 746-5202-5, and by a grant from the Dutch Foundation "Bene Volentia".
The experiments were carried out with the technical assistance of Mrs Harriet O'Connor and Miss Jean Cavallaro. Mr M.A. van 't Hof assisted in the statistical analyses. The figures were designed by Mr W.P.J. Maas. The manuscript was prepared for printing by Miss Else Kuin.

C O N T E N T S

CHAPTER 1

GENERAL INTRODUCTION AND PROBLEM STATEMENT

The relation between experimental and clinical transplantation has two aspects. On the one hand, the development of current clinical transplantation has evidently resulted from findings obtained in animal experiments; on the other hand, it is precisely in the clinical setting that the problems posed by tissue transplantation have regained topical interest, and have given a fresh impetus to experimental research on the transplantation reaction. However, the situations which arise in clinical organ transplantations are so complex, and so difficult to reproduce, that new approaches to improved therapy can be explored only in animal experiments, in which an attempt can be made to reduce them to their component parts, and measure them in a reproducible way.

Our knowledge of the transplantation phenomena largely originates from experimental research on malignant tumours in mice. It has long been known that a tumour which has either occurred spontaneously or has been induced by means of carcinogenic substances, is usually rejected 10-12 days after its transplantation into a non-syngeneic host. This rejection is based on the recognition, by the host, of the histocompatibility antigens present in the tumour (Gorer 1937). These histocompatibility or transplantation antigens can be defined as genetically determined factors present in a transplant, which - if not present in the host - evoke an immunological response that can lead to rejection of the transplanted tissue. Normal tissues are rejected by the same mechanisms as malignant tumours.

In most cases the contact with foreign tissue results in a dual reaction. A cell mediated immune response occurs, which shows the characteristics of a delayed hypersensitivity reaction. As well a humoral response occurs with the synthesis of

circulating antibodies. Our understanding of the interrelation between these two responses is still limited. It is beyond doubt, however, that both mechanisms can play an important role in the rejection of foreign tissue.

In the cellular immune response, specifically activated lymphocytes are formed: so-called "killer"cells which migrate to the graft and cause destruction of its cells. This cytolytic action may be effected through cell-bound antibodies localized on the external surface of the lymphocytes (Amos and Koprowsky 1963).

The humoral response likewise plays a role in the transplantation reaction. With reference to a recent survey of the relevant literature (Winn 1970), we may confine ourselves to the statement that the action of humoral antibodies in vivo can be widely diverse. Experimental and clinical experience has shown that the presence of circulating antibodies against histocompatibility antigens of a graft, for example, a human kidney allograft, can lead to hyperacute rejection. It is also highly probable that humoral antibodies can play a role of importance in chronic graft rejection. We find a counterpart of this in a phenomenon known as immunological enhancement, which can be defined as prolonged survival of a graft in the presence of antibodies directed against transplantation antigens of the graft (Kaliss 1969). Specific antibodies can be passively administered to the recipient by injection, or their presence be effected by active immunization of the host with the same antigens as will be present in a subsequent graft.

Since our interest in the phenomenon of enhancement has prompted the study to be presented, it seems useful first to discuss it in some detail. This phenomenon, too, was first demonstrated in studies with transplantable tumours. It was found that some tumours paradoxically showed enhanced growth despite prior immunization of the recipient to that particular tumour. This was most frequently observed with tumours which were relatively insensitive to the lytic action of antibody and complement in vitro; these were mainly sarcomas. Lymphoid tumours, on the other hand, were found to be readily destroyed by antibody and complement in vitro, and it was very difficult to induce enhancement of the growth of these tumours in vivo. Studies by Winn (1960) and Möller and Möller (1962) demonstrated that the sensitivity to antibody is related to the concentration of antigenic sites on the cell surface. This leads to the conclusion that the enhancement phenomenon can be induced more readily if the concentration of antigenic sites with which the antibody reacts, is lower.

It should be emphasized that the antiserum must contain antibodies against all the transplantation antigens present on the donor cells and absent from the recipient cells; otherwise the antigens not "covered" are able to induce graft rejection. It is therefore difficult to induce enhancement when the genetic disparity between donor and recipient is great, i.e. when many antigenic differences exist. This is probably why it has proved so difficult to achieve significant enhancement of grafts of normal tissue, particularly skin grafts.

It has recently been demonstrated that it is possible to induce enhancement for skin grafts in mice if a genetic model is chosen in which antigenic differences are confined to only a few specificities (McKenzie, Jeekel, Koene and Winn 1970). This result has raised the question of the concentration and distribution of transplantation antigens on the cells in these mouse strains. The study to be presented makes an attempt to improve our insight into this question on the basis of in vitro investigations.

Studies which Möller (1961) made in vitro with the aid of fluorescein-labelled antibodies, disclosed that the antigenic determinants to which the antibodies bind themselves, are localized on the external cell membrane. This antigen-antibody reaction as such, however, does not lead to cell destruction. This final step requires the presence of complement: a system of nine serum factors. Activation of this complement system gives rise to a lesion in the cell membrane, as a result of which the separation between intracellular and extracellular space is disrupted, and the cell destroyed. This lesion was visualized electron-microscopically in the splendid study published by Borsos, Humphrey and Dourmashkin in 1964.

The effect of complement is undoubtedly in part dependent on the geometric relations between the antigenic determinants on the cell membrane. Additional factors of significance are the nature of the antibody bound to the antigenic determinant as well as the origin of the complement. Effects observed with antibodies of the IgM type differ from those seen with antibodies consisting of IgG. There are sound reasons to assume that guinea pig complement requires more antigenic sites for its effect than rabbit complement. In principle, these differences afford the possibility of obtaining information on the distribution of antigenic determinants on the cell membrane by studying the reactions in systems with varying concentrations of antibodies and complements. Moreover, they make it possible to establish whether antigenic determinants of different specificity can interact with regard to the binding of specific antibodies and the activation of complement.

Studies of the abovementioned effects have so far been made largely with the aid of the so-called haemolytic system, in which the destruction of red cells in response to red-cell antibody and complement is investigated. The advantage of this system is that it is relatively simple to measure the degree of cell destruction by the amount of haemoglobin released. Winn in particular has made exhaustive studies of this system, and the study to be presented is an extension of his work (Winn 1965) to a system which is a closer approximation of the transplantation reaction. Specifically, the erythrocytes have been replaced by nucleated lymphoid cells in our study, and the red-cell antibodies by antibodies specifically directed against transplantation antigens. It is by no means a matter of course that the results of studies of the haemolytic system should apply also to cytolysis of nucleated cells. It is possible that these cells are capable of dealing with the damage caused by complement by means of a restorative mechanism of an obscure nature (Chambers and Fell 1931, Müller-Eberhard, Polley and Nelson 1966). It is therefore quite possible that the kinetics of the cytolysis of nucleated cells differ from those of the red blood cells.

A full understanding of this question remains an illusion for the time being; and this study is merely an attempt to offer a model for this type of research, thus making an modest contribution to the knowledge so far gathered.

CHAPTER 2

TRANSPLANTATION GENETICS OF THE MOUSE

The mouse constitutes an excellent model for transplantation research, because large numbers of high inbred strains are available with a constantly homozygous genetic pattern. Moreover, the transplantation genetics of the mouse have been studied in detail, and the various antigens have been clearly defined. The transplantation or histocompatibility antigens are determined by genetic factors: the histocompatibility genes. The sites at which these genes are localized on the chromosome are known as histocompatibility loci or H-loci.

For a good understanding of the model used in this study, the method used to identify the various H-loci should first be briefly discussed. This method is based on the genetic principle of co-isogenicity, and was evolved by Snell (1948, 1958). The co-isogenicity concept postulates identity between two individuals except for one genetic locus. If the locus by which the individuals differ is a H-locus, then grafts exchanged between these two individuals are bound to be rejected. Pure co-isogenicity can occur only as a result of mutation. By evolving an ingenious breeding system and using injections of tumour tissue, Snell developed a method which ensures very close approximation to co-isogenicity between two inbred mouse strains.

The principle of this breeding system can be described as follows. A start is made with two inbred strains, A and B, which are assumed to possess different alleles on the H-locus:alleles A and B. Both strains being homozygous, strain A animals carry two alleles A (AA), while strain B animals carry two alleles B (BB). A strain A tumour will grow in an A animal and ultimately kill it, for there are no significant antigenic differences between the strain A tumour and the strain A animal. A strain B animal, however, will reject this tumour because it possesses

the allele B and therefore recognizes the tumour, which contains the allele A, as foreign. On the basis of injections of tumour tissue, therefore, animals which carry a different allele can be distinguished. The purpose is to incorporate the chromosomal segment which contains the allele B, in animal A while leaving the remainder of the strain A genome unchanged. To achieve this, A and B are crossed and an F_2 generation is made (fig. 1).

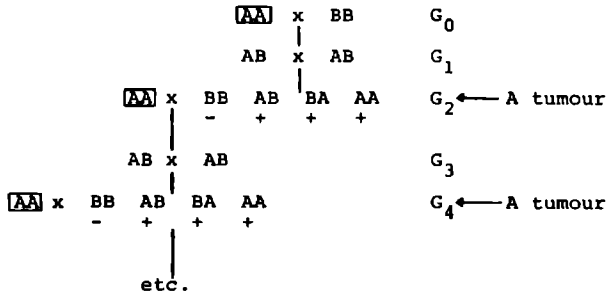


Fig. 1 Production of a congenic resistant line (see text). AA and BB refer to the histocompatibility alleles of strain A and strain B.
+ = susceptible to strain A tumour
- = resistant
G = generation

When strain A tumour tissue is injected into all F_2 animals, only the BB animals will survive. These survivors are backcrossed to the parental strain A, and all subsequent even generations are inoculated with the A tumour. As a result of the repeated backcrosses to the strain A animals, all genetic information of strain B is lost and replaced by the strain A genome. Only the histocompatibility allele B remains intact because this is the gene for which selection takes place. After 12-14 generations the surviving animals will be virtually identical to strain A except for the histocompatibility gene B. In this generation, two surviving animals are then crossed, and the co-isogenic strain is obtained.

The strains are not purely co-isogenic in the strict sense, for in the above described system of breeding, traits which are very closely linked to the allele B can be transmitted with it to the newly established strain. This is why Snell introduced the term congenic, instead of co-isogenic. The newly established strains are called congenic resistant strains. The abovementioned new strain is designated A.B. A is the symbol of the tumour-donating strain, and B that of the resistant strain in the original cross*.

An example of a natural congenic system is the histo-incompatibility between males and females of the same inbred strain. The Y chromosome contains a weak transplantation antigen. On the basis of this single difference, grafts from males to females are rejected in some inbred strains. This, however, is only one-way histo-incompatibility. Female grafts which lack the Y-linked antigen, are not rejected by males of the same inbred strain.

The development of these congenic resistant strains has made it possible to identify the histocompatibility genes and study them separately. It was found that the transplantation antigens can be divided into two groups. There are strong transplantation antigens, which give rise to an intensive transplantation reaction with, for example, rejection of a skin graft within 10-15 days. The other group comprises the less intensive transplantation reactions with graft rejection within 15-300 days.

The genetic information for the strong antigens is localized in a single chromosomal region: the histocompatibility-2 locus (H-2 locus) on the IXth linkage group. More than 20 alleles of this locus are now known, which are designated $H\text{-}2^a$, $H\text{-}2^b$, etc. We know, chiefly from serological studies, that each allele as such is complex and determines a varying number of antigenic specificities. These are designated H-2.1, H-2.2, etc. Some 40 specificities are known. Many antigenic specificities are shared by several alleles, but some others (private antigens) are present on only a single allele.

Recombination studies have demonstrated that the genetic determinants of the antigenic specificities are arranged on the chromosome in a particular linear order (Shreffler 1970). The H-2 region proves to consist of 5 subregions which can be separated by crossing over. The genetic determinant of one particular

* For the sake of convenience it was assumed in the example that both the A and the B strain were inbred. However, the B strain need not necessarily be an inbred strain. The same result can be obtained with a non-inbred strain because selection takes place only for the H-locus.

antigen is always localized in the same particular subregion. The subregions localized at the two ends of the H-2 locus, have been most fully studied because due to their relatively large distance apart, they are most frequently involved in recombinations. These two subregions are known as D end and K end. The antigenic specificities governed by these subregions are known as D end antigens and K end antigens, and the antibodies which correspond with these antigens are known as anti D end and anti K end antibodies.

Snell and co-workers have made the greatest contribution to the analysis of the genetic structure. The data on the antigenic constellation are recorded on the H-2 chart, which is updated at regular intervals (Snell, Hoecker, Amos and Stimpfling 1964, Shreffler and Snell 1969).

The weak or non H-2 transplantation antigens are localized in different linkage groups. In the mouse, 14 non H-2 loci are known, which are designated H-1, H-3, H-4 etc. At most of these loci, too, a number of allelic forms are present, e.g. $H\text{-}1^a$, $H\text{-}1^b$. etc. As we mentioned, a weak transplantation antigen is found on the Y chromosome. A minor histocompatibility locus is likewise found on the X chromosome. Although the non H-2 antigens are weak antigens, combinations of various non H-2 antigens can have a cumulative effect so that an intensive transplantation reaction nevertheless occurs (Graff, Silvers, Billingham, Hildemann and Snell 1966).

In view of the above data it is evident that two groups of congenic resistant strains can be produced. When the parental strains differ on the H-2 locus, a H-2 congenic strain will be bred. In order to obtain a non H-2 congenic strain, the parental strains should be of the same H-2 type.

Immunization of suitable combinations of H-2 congenic strains has made it possible to prepare antisera which contain antibodies directed against a limited number of antigenic specificities. With these, a further analysis of the H-2 locus can be carried out. Most of these antisera are prepared at the Jackson Laboratory, Bar Harbor, Maine. The characteristics are compiled by Snell (1968).

CHAPTER 3

THE ACTION OF COMPLEMENT

Introduction

The complement system has been defined by Mayer (1965) as "a cytocidal reaction system which is activated by antigen-anti-"body complexes or aggregated γ-globulin or by certain other "materials".

The system consists of nine serum factors which interact in a definite sequence. Mayer's definition is too limited, as it has become clear that a number of other reactions are likewise mediated by complement, even though not all nine components are required for this purpose. In this context we are alluding to such phenomena as immunophagocytosis, immune adherence, chemotaxis and anaphylaxis. These phenomena are not within the scope of this study and need not be discussed here. For reviews, the interested reader may be referred to Mayer (1961), Lachmann (1968), Pondman (1969) and Audran (1970).

Our attention focuses on the mechanism of the cytocidal action of complement. As mentioned earlier, the reaction has been most fully studied in a system consisting of sheep red cells, anti-red cell antibody (which is in fact an anti-Forsmann haemolysin) and guinea pig complement. It has been demonstrated that the principles of complement activation in this system also apply to other systems, e.g. bacteriolysis (Muschel 1965) or immune lysis of nucleated cells (Ross and Lepow 1960).

Activation of complement

The course of complement activation in relation to cytolysis is shown schematically in fig. 2. The antigenic site (S) on the erythrocyte is first sensitized with the antibody (A).

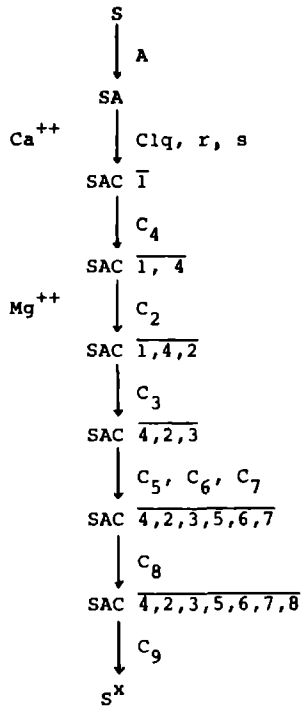


Fig. 2 Schematic representation of the mechanism of complement activation.
S = antigenic site on cell;
A = antibody; C_1, C_2 etc. = complement components;
C $\overline{1}$ etc. = activated form;
S^x = membrane lesion.

At the antigenic site the antibody combines with a specific part of the molecule, the so-called Fab fragment. This fragment can be separated from the total molecule by treatment with papain.

The remainder, the so-called Fc fragment, is responsible with an intact molecule for complement activation. For, if Fab fragments without Fc fragment are combined with the antigenic site, then complement fixation is greatly reduced.

The first complement component (C1) is a macromolecule which can be artificially split up into three subcomponents: C1q, C1r and C1s. The division is achieved by extracting calcium with the aid of chelating agents. Component C1q combines with the Fc fragment of the antibody molecule. Once this combination is achieved, component C1s (a pro-esterase) is transformed into C1-esterase under the influence of component C1r which acts as protease. Calcium ions are required for this reaction. Levy and Lepow (1959) demonstrated that normal serum contains an inhibitor of C1-esterase. This factor is able to inhibit the enzymatic function of C1-esterase, thus preventing spontaneous complement activation. In the vicinity of a cell sensitized with antibody, however, the concentration of complement becomes so high that the inhibitory effect on C1-esterase is overwhelmed. Moreover, the velocity of the inhibitory reaction is lower than that of the C1 activation.

C1-esterase will bring C4 in an activated state. During this activated state, C4 is able to combine with receptors on the cell membrane or on the antibody. C4 is active only during a short time. If it does not combine with a receptor, it is transformed into an inactive form (C4i). Most of C4 combines with the cell membrane, while only a small amount is adsorbed by $\overline{SAC1}$. The mechanism underlying this process is obscure.

The formation of $\overline{SAC1,4,2}$ takes place in two consecutive stages. In the first stage, C2 is adsorbed onto $\overline{SAC1,4}$; this reaction occurs only in the presence of magnesium ions. In the second stage, the adsorbed C2 is split into two parts by C1-esterase. The active part ($\overline{C2}$) combines with $\overline{SAC1,4}$ whereas the inactive part (C2i) enters the fluid phase, The intermediate $\overline{SAC1,4,2}$ is not very stable. The component C2 is readily released from the complex and can be found in the fluid phase in an inactive form called $C2a^d$. The half-life of $\overline{SAC1,4,2}$ is about 10 hours at 0^oC, and about 10 minutes at 37^oC. The complex $\overline{SAC1,4}$ which remains after release of $\overline{C2}$ can react with a new C2 component and once again form a $\overline{SAC1,4,2}$ complex.

Once this stage is attained, $\overline{C1}$ is no longer required for the reaction. It can be easily eluted from the complex with EDTA. The behaviour of the complex $\overline{SAC4,2}$ is the same as that of $\overline{SAC1,4,2}$. This is why the symbol C1 is discarded from this stage on.

The $\overline{C4,2}$ complex acts as an enzyme which has C3 as its natural substrate. It is also known as C3 convertase. It is

by the action of this enzyme that C3 is activated. The activated C3 can become fixed, or it remains free and decays to an inactive form (C3i). One site of $\overline{C4,2}$ is able to catalyse the fixation of several hundred C3 molecules to receptor sites on the cell membrane, but it seems likely that only C3 molecules bound in close proximity to the $\overline{C4,2}$ can induce haemolysis.

The action of C5, C6 and C7 leads to the formation of a stable intermediate. There is no decay at 37°C. When the subsequent complement components are added after several hours' incubation at this temperature, lysis still occurs. C5, C6 and C7 act as a functional unit in their reaction with $SA\overline{C4,2,3}$, but the exact nature of this reaction is not clear. It is uncertain whether any part of the C5,C6,C7 complex becomes fixed to the cell. Most of it is released in the solution as a complex with chemotactic properties.

C8 reacts with the stable intermediate and is bound on the cell membrane. In the next step, C9 reacts with the active site of C8. This results in a lesion of the cell membrane ($S^{\blacktriangledown}$). As we mentioned, these lesions can be electron-microscopically visualized as small "holes" (Borsos et al. 1964). These holes are considered to be local changes in the lipid layer of the cell membrane. Once $S^{\blacktriangledown}$ is formed, the cell lyses spontaneously in the absence of any serum factor. The chemical process by which the lesion of the cell membrane is brought about, is still obscure.

We have thus described the phenomenon of cytolysis in terms of reactive sites. Many such sites are usually present on the surface of one cell. Mayer (1965) analysed the kinetics of the different steps in complement activation and, on the basis of his findings, developed his "single hit theory". This theory postulates that the production of only one $S^{\blacktriangledown}$ on a cell is sufficient to induce its lysis. The successive hits which may occur on one cell do not interact to induce cytolysis, but are independent events. Borsos et al. (1964) demonstrated that the number of damaged sites on the cell as predicted from the single hit theory, was remarkably close to the number of holes detected by electron microscopy.

The principle of complement activation is probably the same for complements from different sources. However, there are species differences which can cause quantitative modification of the cytocidal action. These will be discussed in later sections of this study.

CHAPTER 4

SPECIFIC DEFINITION OF THE PURPOSE OF THIS STUDY

Cytotoxic cell destruction in vitro is a measure of the relationship between cell surface antigens, specific antibody, and complement. The susceptibility of cells to the destructive action of antibody and complement is related to the concentration of corresponding antigenic sites on the cell surface. Studies by Winn (1960, 1962) and Möller et al. (1962) have shown that cells susceptible to the lytic action of antibody and complement, can absorb much larger amounts of these antibodies than resistant cells.

The source of complement also plays an important role, for it has been established that mouse cells sensitized with alloantibody are more efficiently destroyed by rabbit complement than by guinea pig complement (Boyse, Old and Thomas 1962, Winn 1965, Haughton and McGehee 1969). The lastmentioned authors suggested that this difference in effect between the two types of complement are caused by the fact that guinea pig complement requires two sites sensitized with antibody to be activated, whereas rabbit complement needs only one.

If the hypothesis advanced by Haughton et al. is correct, then guinea pig complement should become more efficient as the number of reactive sites per unit of cell surface is increased (always assuming that the antigens are evenly distributed on the cell surface). This situation might be achieved by sensitizing the cells with combinations of antisera which contain different specificities, i.e. which are directed against more than one antigenic determinant. Two additional requirements must be met as well, namely:

1) the complement should not discriminate between antibodies of different specificity;

2) the reactive sites of different specificities should be localized sufficiently close together to permit the complement activation.

The first of these two requirements can be assumed to be fulfilled. We may regard complement activation as a sort of "final common pathway" to effect lysis of the cell, and as independent of the antigen-antibody reaction involved. In fact, the antigenic determinant against which the antigen is directed needs not even to constitute an integral part of the cell membrane. Middlebrook demonstrated in 1950 that erythrocytes coated with soluble antigen from, for instance, tubercle bacilli, can be haemolysed by addition of complement and antibody specifically directed against the bacilli.

The situation is much more complicated with regard to the second requirement. For the literature comprises reports on synergistic as well as on antagonistic or blocking effects of polyspecific sera. Möller et al. (1962) prepared antisera against a single type of tumour cells in three different mouse strains. The cells were not lysed by anyone of the antisera used alone, but were by a combination of the three antisera. A few years later, Möller and Eklund (1965) also demonstrated a synergysm of anti-ABO and anti-rhesus antibody in the lysis of human lymph-node cells. Rosse and Parker (1968) demonstrated that human erythrocytes sensitized with three anti-rhesus sera containing different specificities, were able to bind complement factor C1a, which was not bound when antiserum against one specificity was used. The source of complement used in all the abovementioned studies was fresh guinea pig serum. Ivasková, Vybíralová, Raue, Démant and Iványi (1969) demonstrated a similar synergism of HLA antibodies. Although they do not specifically state this in their publication, it seems likely that they used rabbit complement in their study. However, they rightly pointed out that their experiments disregarded possible anticomplementary effects as well as differences in molecular classes of antibody between the sera used. On the other hand Boyse, Old and Stockert (1968) and Kristofová, Lengerová and Rejzoková (1970) demonstrated with mouse cells that a single antibody specificity can counteract the absorption of another antibody, probably by a blocking effect. Both groups of investigators made use of rabbit complement. In 1968, Cresswell and Sanderson described a blocking effect of H-2 antibodies in a cytotoxic test in which they used guinea pig complement.

These differences must be assumed to depend on the geometric localization of the antigenic sites: if these are very close together on the cell surface, then we may assume that an antigen-antibody complex constitutes a steric obstacle against the binding of antibody to a nearby antigenic determinant. If the anti-

genic sites are so distributed as to give no access to complement individually, but only collectively, then synergism may be expected. Finally, the sites may be spaced so far apart that there can be neither a reciprocal unfavourable influence nor an improved access to complement upon collective activation.

The study by Boyse et al. (1968) demonstrated that blocking occurred only if combinations of anti D end antibodies were used. No blocking effect was demonstrable, however, with mixtures of anti D and anti K specificities. These results suggest that, on the cell surface, the distance between D end antigenic determinants of different specificity is smaller than the distance between D end and K end antigens.

In order to study these blocking and synergistic effects in the system we used, we first carried out titrations, at an excess of two types of complement (rabbit and guinea pig complement), with optimally monospecific antisera; next we combined these antisera to polyspecific antisera of known composition. The sera were so chosen as to ensure that sera with only anti D end antibodies as well as sera with only anti K end antibodies were represented in the material. The results of this investigation are presented in chapter 6 under the heading "antiserum titrations". In addition, we carried out titrations with the two types of complement at constant antibody concentrations. The data thus obtained are presented in chapter 7 under the heading "complement titrations".

CHAPTER 5

THE TEST SYSTEM USED IN THIS STUDY

The test system used in this study can be described on the basis of the schema:
Antigen-bearing target cell + antibody $\xrightarrow{\text{complement}}$ lysis of target cell.

Tumour cells of the lymphoid leukaemia type (L1210 cells) were used as antigen-bearing target cells. The tumour in question was originally induced in the DBA/2 mouse strain by skin paintings with 0.2% methylcholanthrene in ethyl ether (Law, Dunn, Boyle and Miller 1949). The tumour can be easily passaged in DBA/2J mice and in (C57Bl/6J x DBA/2J)F_1 hybrids. We usually employed the latter strain.

Ascites develops within a few days of intraperitoneal injection of 10^6 tumour cells. The ascites fluid is rich in tumour cells and can be simply used for preparation of a cell suspension. These cells are quite suitable for use in a cytotoxic test and prove to be much less vulnerable to non-specific trauma than normal mouse lymphoid cells.

The transformation of normal tissue into tumour tissue is associated with acquisition of new antigenic determinants, including tumour specific transplantation antigens (TSTA). Foley was the first to demonstrate the immunological function of TSTA in 1953. When he inoculated a mouse with tumour tissue and removed the inoculate before the animal succumbed to progressive tumour growth, a second inoculate proved not to grow: the mouse had become immune to the tumour, and this immunity proved to be specific because other methylcholanthrene-induced tumours were not rejected.

The TSTA formed in chemically induced tumours prove to have a different antigenic specificity in virtually all cases. This also applies to tumours induced by means of the same carcinogen in different animals of the same inbred strain (Klein and Klein 1962, Holmes, Morton, Schidlovsky and Trahan 1971).

Since the DBA/2 mouse carries the $H\text{-}2^d$ allele, L1210 tumour cells can be expected to possess the $H\text{-}2^d$ antigens. To eliminate the possibility of the cell losing one or several $H\text{-}2^d$ antigens in its transformation to tumour cell, an earlier study had focused on the question whether $H\text{-}2^d$ antigens could be demonstrated on the L1210 cell (Koene, McKenzie, Painter, Sachs, Winn and Russell 1970). The H-2 specificities 3, 4, 8, 13, 28 and 31 were demonstrated. All antigenic specificities which we wish to examine in this study,were present.

The ascites fluid formed following intraperitoneal injection of tumour cells, is usually highly haemorrhagic. The erythrocytes must be removed before use. For this purpose the peritoneal cavity of the ascites-bearing mouse is washed out with a Tris-NH_4Cl solution, and the cell suspension thus obtained is then incubated at 37°C for 10 minutes. This method, which is virtually the same as that described by Boyle (1968), is used to lyse the red cells. The cells are then centrifuged in a refrigerated centrifuge (4°C) at 200 g for 5 minutes, and suspended in Hank's balanced salt solution (BSS) (Hank and Wallace 1949), without sodium bicarbonate, to which foetal calf serum has been added in advance to a concentration of 5%. The pH of this medium is 6.8. The final concentration of cells is adjusted to 2×10^7 cells per ml.

A number of allo-antisera were used as antibody in this study. All were specifically directed against one or two $H\text{-}2^d$ antigenic determinants. A survey of the sera used is presented in Table I.

TABLE I: SPECIFICITIES OF ALLO-ANTISERA TESTED

Antiserum	H-2 specificities present	H-2 specificities directed against $H\text{-}2^d$ antigens
(B10 x A) anti B10.D2[x]	31	31
(AKR.M x C3H.SW) anti B10.A	4	4
(B10 x A.SW) anti B10.M	8,9	8
(B10.Br x 129) anti B10.AKM	13,30	13
(A.CA x B10.Br) anti A.SW	3,19,28	3,28

[x] ascites fluid (see text)

With one exception, the allo-antisera were obtained from the Transplantation and Immunology Branch of the National Institute for Allergic and Infectious Diseases (Bethesda, Maryland), which is provided with tested sera by Dr G.D. Snell (Jackson Laboratory, Bar Harbor, Maine). Their characteristics are compiled in the "Catalog of mouse allo-antisera 1968".

The hetero-antiserum we used, was kindly supplied by Dr D.H. Sachs (Department of Surgery, Massachusetts General Hospital, Boston).

The preparation of antisera is best discussed with reference to the anti H-2.31 allo-antiserum which we ourselves prepared in the laboratory.

The principle of preparation is based on immunization of the recipient with donor cells possessing only a few antigenic determinants not present on the recipient's tissues. This ensures the formation, in the recipient, of antibodies directed only against one or a few known specificities of the donor.

Anti H-2.31 antiserum was induced in female (C57Bl/10ScSn x A/Jax)F_1 = (B10 x A)F_1 hybrids by multiple intraperitoneal injections of B10.D2 lymphoid cells. The cells were obtained from lymphnodes, spleen and thymus, and made into a cell suspension by pressing the tissue through a 60-gauge stainless steel mesh into Hank's BSS.

The antigenic determinants of the mouse strains used are presented in table II, which shows that the only antigenic determinant present in the B10.D2 strain and absent from the A/Jax strain, is specificity H-2.31.

TABLE II: H-2 ANTIGENIC SPECIFICITIES OF THE THREE MOUSE STRAINS USED FOR RAISING ANTI H-2.31 SERUM

Strain	H-2 Allele	Antigenic specificities
A/Jax	a	1 3 4 5 6 8 10 11 13 23 25 27 28 29
C57Bl/10	b	2 5 6 22 27 28 29 33
B10.D2	d	3 4 6 8 10 13 27 28 29 (31)

But in addition to H-2 differences there could also be non H-2 differences between these two strains. Our effort to eliminate these makes use of the fact that the B10.D2 strain is congenic with the C57Bl/10 strain, that is to say: these two strains differ only at the H-2 locus. The hybrid (B10 x A)F_1 should therefore carry all non H-2 antigenic determinants of the B10.D2 strain, so that immunization of the (B10 x A)F_1 strain with B10.D2 cells should lead to formation of antibodies specifically directed against specificity H-2.31.

The majority of investigators obtain the antiserum by bloodletting from the tail vein or from the retro-orbital venous plexus. Only minute amounts of antiserum can thus be collected

per mouse. The technique we use, which was first described by Munoz in 1957, enables us to produce larger amounts of antibody in a simple way.

We injected lymphoid cells intraperitoneally in complete Freund's adjuvant (CFA). It is possible that a more intensive humoral response occurs as a result of its addition (Freund 1951). The injections were given weekly. Marked ascites was found to form in the test animals after a few weeks. In the ascites fluid, antibody was demonstrable at a titre which equalled that of the serum. The procedure can be described in detail as follows.

1. Recipient (B10 x A)F_1 is injected every 1-2 weeks with 1-2 x 10^7 lymphoid cells of B10.D2.
2. Intraperitoneal injections of 0.25 ml in CFA are given (equal volumes of cells and adjuvant).
3. Ascites forms after 4-5 injections and is tapped every 1-2 weeks.
4. Further immunization with cells without adjuvant.

It should be pointed out that Davies (1969) demonstrated a weak reaction of H-2.31 serum with F/St ($H\text{-}2^n$) mouse cells as well as with DBA/1($H\text{-}2^q$) cells. This might imply that this serum contains yet another specificity, the corresponding antigen of which should be present in $H\text{-}2^d$, $H\text{-}2^n$ and $H\text{-}2^q$. Davies designated this specificity H-2.34. In the antiserum we prepared, too, a weak reaction with DBA/1 cells was demonstrable. Absorption of the serum with 10^8 DBA/1 cells, however, proved incapable of abolishing the anti-31 activity. The titre with B10.D2 cells remained virtually unchanged after absorption. This means that DBA/1 possesses no antigen 31 and that the reaction with the serum should therefore be based on another specificity. Actually, therefore, the antiserum contains more than one specificity, and should be designated H-2.31,34. The reaction against H-2.34, however, is weak. With all subsequent references to anti H-2.31, it should be borne in mind that this serum contains at least one more specificity. Other unknown specificities may be present, but the likelihood of finding these is decreased by using F_1 hybrids as recipients in the immunization procedure. Because these animals are heterozygous, more antigens will be represented and hence the probability of finding antigenic differences between donor and recipient should be less than in cases where homozygous recipients are used.

As we mentioned, the hetero-antiserum used was supplied by Sachs. In a splendid study (Sachs et al. 1971), he demonstrated that hetero-antisera can recognize not only "species-specific" antigens but can also detect H-2 allo-antigens. Sachs prepared

a rat anti B10.D2 lymphocyte serum by intraperitoneal injection of B10.D2 lymphocytes into Lewis rats, followed by weekly booster injections of these cells. The serum sample used in this study had been obtained 8 weeks after the first injection. Before use, it had been heat-inactivated at 56°C for 45 minutes. This abolishes complement activity by inactivating the heat-unstable components C1 and C2 (Kabat and Mayer 1961). This serum showed an equal activity against B10.D2($H\text{-}2^d$) and B10.Br($H\text{-}2^k$) lymphocytes. The cytotoxic activity against B10.Br cells was abolished by prior absorption with 6×10^8 B10.Br cells per ml serum. After this absorption, however, cytotoxic activity against B10.D2 cells persisted; further absorption with B10.Br cells likewise failed to abolish this activity. It was concluded that the serum contained antibodies detecting $H\text{-}2^d$ allo-antigens and mouse "species-specific" antigens, and that consequently H-2 specificities can be detected across a species barrier. After absorption with (B10 x A)F_1 lymphoid cells it was shown that this serum was monospecific, containing antibodies reactive only with H-2.31 (Sachs et al. 1970). Both the non-absorbed and the absorbed serum were used in our study.

The molecular class of the antibodies used can be of great importance in complement activation. Winn (1965) demonstrated that guinea pig complement can be activated more readily by IgM than by IgG antibodies. This fact is probably related to a difference in complement fixation by the two classes of antibody. Borsos and Rapp (1965) suggested that a single molecule of IgM suffices to activate guinea pig complement, whereas two adjacent molecules of IgG are required to obtain this effect. It is to be expected that the cell must be sensitized with many IgG molecules before a situation arises in which two molecules are in juxtaposition. It was found that at least 100 IgG molecules are required to fix guinea pig complement, for which purpose only a single IgM molecule sufficed (Humphrey and Dourmashkin 1965). All these studies were carried out with the aid of rabbit antibody, guinea pig complement and sheep red cells. A study by Andersson, Wigzell and Klein (1967), however, showed that the same applies to mouse alloantibody, guinea pig complement and nucleated cells. Although these authors all seem to agree that IgM is more efficient with guinea pig complement, much remains to be clarified. It was shown in a recent report by Frank and Gaither (1970a, 1970b) that IgM is not always more active in the haemolytic system. This is the case only if excess antibody is used and if the reaction is carried out at 37°C. In a limited antibody system at 37°C, IgG became more efficient at certain antiserum dilutions. They also demonstrated that, at 4°C, the efficiency of IgM fell to very low levels, whereas IgG activity remained relatively unchanged.

It therefore seems important to establish the class of antibody present in the antiserum before drawing conclusions on activity, especially when comparing reactions with complement from different sources.

In the present study, the molecular class of the antibodies used was established with the aid of 2-mercapto-ethanol. Treatment of antisera with this substance can be presumed to destroy IgM activity (Uhr and Finkelstein 1963). Sera were incubated in a final concentration of 0.1 M 2-mercapto-ethanol for 30 minutes at 37°C. After treatment the sera were dialysed against phosphate buffered saline to remove excess 2-mercapto-ethanol. The cytotoxic activities of the sera did not change after this treatment. In view of this finding it is likely that all antisera contained mainly IgG antibodies.

The complement used in this study came from two sources: guinea pig and rabbit. As we pointed out, there are substantial differences in activity between complements from certain species. Boyse et al. (1962) demonstrated that mouse lymphoid cells sensitized with allo-antibody were much more effectively lysed by rabbit complement than by guinea pig complement. Later studies by Winn (1965) and by Haughton et al. (1969) confirmed these findings. The lastmentioned authors explained the difference in efficiency between the two types of complement by suggesting that guinea pig complement probably requires two antigen-antibody complexes to become activated, whereas a single complex is sufficient for rabbit complement. These problems will be further discussed in subsequent chapters.

Fresh lyophilized guinea pig serum was obtained from Difco Laboratories, Detroit, Michigan. Several lyophilized samples were reconstituted to the original volume with distilled water, pooled and stored at -70°C in one ml aliquots.

Fresh frozen serum from New Zealand rabbits was used as a source of rabbit complement. Rabbit serum often contains a natural circulating antibody against mouse cells, which makes it toxic in the test. It was therefore frequently necessary to test sera from 10-20 rabbits before a non-toxic serum was obtained. Some investigators have tried to remove the circulating antibody by absorption with mouse lymphoid cells at 0°C. In our experience, however, the absorption also resulted in greatly reduced complement activity which made the serum unsuitable for titrations. This is why in this study we only used non-absorbed sera which were found non-toxic to mouse lymphoid cells.

Lysis of target cells can be measured by a number of methods. The oldest and most commonly used technique is the trypan blue dye exclusion method, first described by Pappenheimer (1917) and later

modified by Gorer et al. (1956). This method makes use of the fact that dead cells take up trypan blue whereas live cells do not. By counting the percentage of stained cells under a microscope, a measure of the number of cells actually killed in a cell suspension can be obtained. The disadvantages of this method are its inaccuracies introduced by subjective errors, and the need to read results within a few hours because, otherwise, the number of dead cells in the controls increases markedly.

For this study we made use of the ^{51}Cr release assay, described by Goodman in 1961. He introduced a technique which employed radioisotopes as indicator system. The cells to be studied are labelled with ^{51}Cr, which is intracellularly bound. When the cell lyses, the isotope is released and, after centrifugation, the amount of radioactivity released can be measured in the supernatant of the cell suspension. With the use of this method, results are reproducible (Sanderson 1964), and the counting need not be done immediately so that several tests can be run simultaneously. Wigzell (1965) demonstrated that the test is as sensitive as the trypan blue method, while its reproducibility is evidently better. Like Goodman (1961), he found that some 75% of the isotope is released upon the death of the cell. The remaining 25% is firmly bound to larger cellular components which precipitate together with the intact cells during centrifugation. Sanderson (1964), who studied the kinetics of the assay in detail, likewise found that 100% cytolysis with the trypan blue method corresponded to 70-80% isotope release.

In different experiments, we used one of two methods for the cytotoxic test: the one stage test and the two stage test. The two methods differ only in that in the one stage test the cytotoxic reaction occurs in the antiserum milieu, whereas in the two stage test the antiserum, after incubation with the target cells, is replaced by another medium. This is done in order to eliminate the anticomplementary activity of mouse serum. In detail, the procedures were as follows.

Labelling of cells. One ml of the cell suspension was incubated at 37°C during 45 minutes with 0.1 ml $Na^{51}CrO_4$ in saline. The isotope was obtained from Nuclear Chicago, cat.no.CJS-1P; 0.1 ml sodium chromate represented 0.1 mC radioactivity. During incubation, the sample was kept in continuous movement to prevent precipitation of cells. After incubation, the cells were washed three times in Hank's BSS in order to remove the free ^{51}Cr. The final volume was adjusted to a concentration of 10^6 cells per ml.

In preliminary studies we used medium 199 as incubation medium (Morgan, Morter and Parker 1950). We found, however, that the cells incorporated twice as much ^{51}Cr when incubated with Hank's BSS to which no $NaHCO_3$ had been added. This affords an obvious advantage: when the ^{51}Cr label is higher, smaller amounts of cells can be used

in the test, thus enhancing its sensitivity. We have not analysed the cause of the higher uptake in Hank's BSS. Since we did not add $NaHCO_3$, it seems probable that it is related to the pH of the solution.

One stage test. We used the method described by Sanderson (1964) with minor modifications: 50 µl antiserum and 50 µl cell suspension (5 x 10^4cells) were incubated with 50 µl complement in a water bath at 37°C during 30 minutes. After 15 minutes' incubation the cells were shaken. After incubation the samples were centrifuged in a refrigerated centrifuge (4°C) at 200 g for 5 minutes. Next, 50 µl of the supernatant was removed and used for measurement of radioactivity in a well-type scintillation counter (Packard Auto-Gamma Spectrometer).

Two stage test. 5 x 10^4cells (50 µl) were sensitized with 50 µl antiserum by incubation at 4°C during 30 minutes. The suspension was then centrifuged and the supernatants were removed simply by shaking out the tubes. No cells were lost in this procedure, as we have demonstrated by control counts of the available radioactivity before and after removal of the supernatants. Next, 50 µl complement and 100 µl of Hank's BSS were added. The subsequent steps were the same as in the one stage test.

Controls. In all tests, the following controls were used.

1. Cell control: 50 µl cells + 100 µl Hank's BSS.
2. Antibody control: 50 µl cells + 50 µl undiluted antiserum + 50 µl Hank's BSS.
3. Complement control: 50 µl cells + 50 µl complement (RC or GPC) + 50 µl Hank's BSS.

The maximum lysis in the control tubes was 15%, but in most cases it was less than 10%.

Calculation of lysis percentage. When repeatedly frozen and thawed, L1210 cells release 80% of the total label incorporated. As we mentioned, this value was found also by Sanderson, who used normal lymphoid cells. We have therefore taken this 80% release as equivalent to 100% lysis. The 80% value may not represent exactly 100% lysis in all serological systems, but in these experiments it has proved to be a convenient (if arbitrary) reference point. The exact manner of calculation is given below.

Total cell label (5 x 10^4cells): A counts per minute (cpm).

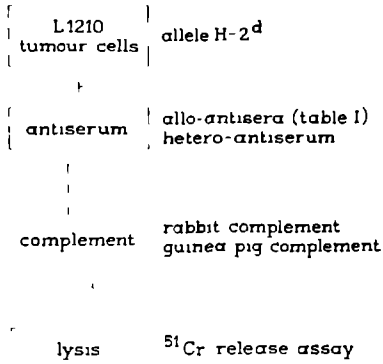
Release at 100% lysis (calculated): $\frac{80}{100}$ x A cpm.

Counts in 50 µl supernatant: B cpm.

Total volume being 150 µl, amount of ^{51}Cr released: 3 x B cpm.

$$\% \text{ lysis: } \frac{\text{amount of } ^{51}Cr \text{ released}}{\text{release at 100\% lysis}} \times 100 = \frac{3 \times B}{\frac{80}{100} \times A} \times 100 = \ldots \%.$$

Reverting to the schema given in the opening sentence of this chapter, the test system used can be summarized as:



CHAPTER 6

ANTISERUM TITRATIONS

All antisera were tested in serial dilutions (1.5 fold or 2 fold steps) with excess rabbit or guinea pig complement, obtained by adding complement in a dilution of 1/8. Mixtures of allo-antisera were prepared by combining equal volumes of separate antisera. The results of each test were plotted on log probit paper. To obtain 50% lysis, a line of best fit was drawn. The antibody titre was expressed in cytotoxic units (CTU) per µl. One CTU is defined as the amount of antibody which gives 50% lysis. An example of the actual calculation is given below.

An antiserum gives 50% lysis in a dilution of, say, 1/100. This means that a dilution of 1/100 contains 1 CTU. The undiluted sample therefore contains 100 CTU. Since 50 µl serum is used in the test, the cytotoxic activity must be $\frac{100}{50}$ = 2 CTU per µl.

TABLE III: EFFECTS OF TWO COMPLEMENT PREPARATIONS ON THE CYTOLYSIS OF L1210 CELLS; CYTOTOXIC ACTIVITIES OF ALLO-ANTISERA TESTED SEPARATELY

Specificities	Cytotoxic activity	
	Rabbit C^{x} (CTU/µl)	Guinea Pig C^{xx} (CTU/µl)
31	12.86	1.16
4	8.30	0.36
3,28	28.18	1.46

[x] Duplicate titrations. Relative S.E.M. 5.8%

[xx] Triplicate titrations. Relative S.E.M. 7.4%

Abbreviations in this and following tables:
C = complement; CTU = cytotoxic units;
S.E.M. = standard error of the mean.

Allo-antibody

The results of the titrations of the separate antisera are presented in table III and fig. 3. It is evident that the cytotoxic activity of all sera is significantly lower with guinea pig complement than with rabbit complement.

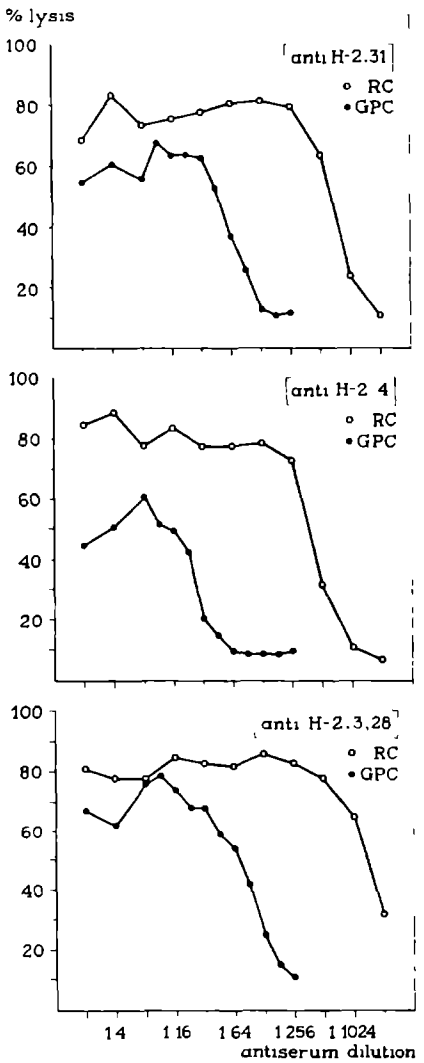


Figure 3: Effects of two complement preparations on the cytolysis of L1210 cells. Antiserum titrations with allo-antisera used separately. Abbreviations in this and following figures: RC = rabbit complement; GPC = guinea pig complement.

The two antisera H-2.8 and H-2.13 showed no activity with guinea pig complement, whereas there was definite cytolysis in the presence of rabbit complement. The results of these titrations are shown in table IV.

TABLE IV: EFFECTS OF TWO COMPLEMENT PREPARATIONS ON THE CYTOLYSIS OF L1210 CELLS. CYTOTOXIC ACTIVITIES OF ALLO-ANTISERA H-2.8 AND H-2.13

Specificity	Cytotoxic activity		
	Rabbit C	Guinea Pig C	
		one stage test	two stage test
	(CTU/µl)	(CTU/µl)	(CTU/µl)
8	2.17	no activity	0.15
13	1.14	no activity	0.11

However, when the same titrations were carried out in a two stage test in which anticomplementary effects were almost completely excluded, these two particular sera showed activity also with guinea pig complement, demonstrating that in these cases anti-complementary factors played a decisive role. Nevertheless, the cytotoxic activity with guinea pig complement remained definitely lower than that with rabbit complement. The titration curves for both sera are presented in fig. 4.

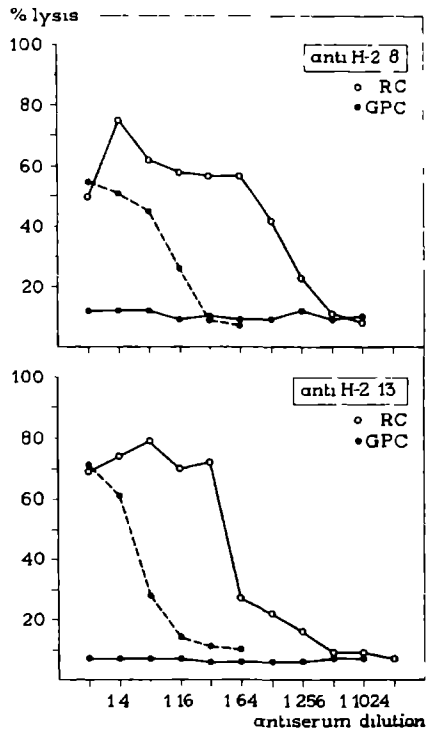


Figure 4: Effects of two complement preparations on the cytolysis of L1210 cells. Antiserum titrations with allo-antisera H-2.8 and H-2.13 (——— one stage test, ------ two stage test).

Mixtures of allo-antibody

The cytotoxic activity of the antiserum mixtures tested, is shown in table V. The actual titrations are given in fig. 5.

TABLE V: EFFECTS OF TWO COMPLEMENT PREPARATIONS ON THE CYTOLYSIS OF L1210 CELLS. CYTOTOXIC ACTIVITIES OF MIXTURES OF ALLO-ANTISERA

Specificities	Cytotoxic activity					
	Rabbit C			Guinea Pig C		
	measured[x] (CTU/µl)	expected[xxx] (CTU/µl)	p†	measured[xx] (CTU/µl)	expected[xxx] (CTU/µl)	p†
31 + 4	8.04	10.58	<0.01	0.52	0.76	<0.01
31 + 3,28	16.72	20.52	<0.02	1.36	1.31	>0.75
4 + 3,28	14.38	18.24	<0.02	1.12	0.91	<0.07
31 + 4 + 3,28	16.23	16.44	>0.75	0.88	0.99	>0.25

[x] Duplicate titrations: relative S.E.M. 5.8%

[xx] Triplicate titrations: relative S.E.M. 7.4%

[xxx] Maximal expected value calculated from cytotoxic units of separate allo-antisera (see text)

† P-value of Student's t test for the differences between measured and expected values.

The maximal expected cytotoxic activity was calculated from the cytotoxic units of the separate sera by taking the sum of these units and dividing this figure by the amounts of sera used in the mixture. For example, the maximal expected value for the mixture of antiserum H-2.31 and antiserum H-2.4 was $\frac{12.86 + 8.30}{2} = 10.58$ CTU per µl in the presence of rabbit complement.

The results presented in table V show that the activities measured for both types of complement were in most instances equal or below the maximal expected values, demonstrating the absence of synergistic effects. In only a single instance was the activity measured significantly higher than that calculated. This was the case with combination 4 + 3,28 with guinea pig complement. The activity measured was 1,12 CTU/µl, while that calculated was 0.91; the difference was weakly significant ($P < 0.07$). In the combination 31 + 3,28 with guinea pig complement the value measured (1.36) was likewise higher than that calculated (1.31),

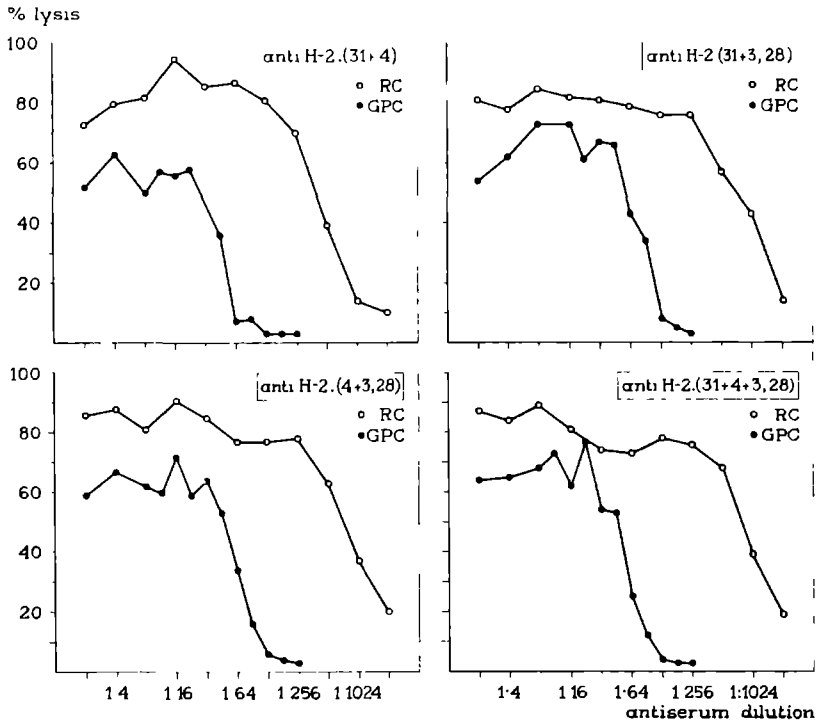


Figure 5: Effects of two complement preparations on the cytolysis of L1210 cells. Antiserum titrations with allo-antiserum-mixtures.

but this difference was not significant ($P > 0.75$). In the combination 4 + 3,28 with guinea pig complement, therefore, a synergistic effect can be suspected. However, another titration with guinea pig complement (31 + 4) disclosed a blocking effect: the activity measured was significantly lower than that calculated ($P < 0.01$). In the combination of three sera (31 + 4 + 3,28), these two effects would seem more or less to cancel each other out: there was no significant difference between the values measured and those calculated.

In all titrations with rabbit complement but one (31 + 4 + 3,28), a significantly lower cytotoxic activity was measured than could be expected on the basis of the results of titrations with the separate sera. This indicates that the sera used in the antiserum mixtures show reciprocal blocking effects when rabbit complement is used.

Hetero-antibody

The results of titrations of hetero-antiserum (fig. 6) again demonstrate that guinea pig complement is less efficient than rabbit complement. This applies to the non-absorbed serum, but also to the serum absorbed with (B10 x A)F_1 cells in advance.

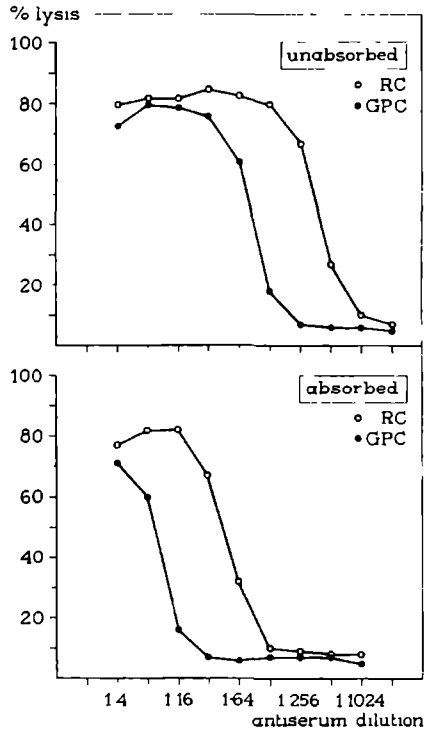


Figure 6: Effects of two complement preparations on the cytolysis of L1210 cells. Antiserum titrations with a hetero-antiserum (rat-anti B10.D2 serum) (two stage test).

After absorption, all sera were noticed to show an increase in anticomplementary activity; this made it necessary to carry out the titrations in a two stage test.

In order to express the relative efficiency of the two complements, we calculated a ratio by dividing the number of cytotoxic units obtained with rabbit complement, by that obtained

with guinea pig complement. Table IV shows that this ratio was 4.94 ± 0.50 for the unabsorbed anti-lymphocyte serum. As we discussed before, mouse species specificities can be removed from the serum by absorption with appropriate mouse lymphoid cells, leaving only strain specific antibodies in the serum.

TABLE VI: EFFECTS OF TWO COMPLEMENT PREPARATIONS ON THE CYTOLYSIS OF L1210 CELLS. CYTOTOXIC ACTIVITY OF A HETERO-ANTISERUM (RAT ANTI B10.D2 SERUM)[x]

Rat anti B10.D2 serum	Cytotoxic activity		Ratio
	Rabbit C (CTU/µl ± S.E.M.)	Guinea Pig C (CTU/µl ± S.E.M.)	RC/GPC ± S.E.M.
unabsorbed	6.95 ± 0.34	1.42 ± 0.04	4.94 ± 0.50
absorbed	0.92 ± 0.18	0.18 ± 0.02	5.01 ± 0.39

[x]Duplicate titrations in two stage test

When this was carried out with (B10 x A)F_1 lymphoid cells, the cytotoxicity with both complements decreased. The ratio, however, remained virtually unchanged: 5.01 ± 0.39 ($P > 0.75$). The relative efficiency of guinea pig complement did therefore not decrease after removal of many specificities by absorption. No distinct synergistic effect between mouse species and H-2 specificities was observed.

Maximal release of ^{51}Cr with rabbit complement and with guinea pig complement

Not only was the cytotoxic activity of the antisera much lower in the presence of guinea pig complement, but the maximal release of ^{51}Cr obtained in the presence of excess antibody was likewise less. It was found that, in all allo-antiserum titrations (fig. 3; fig. 5), the maximal release with guinea pig complement was 10-20% lower than that with rabbit complement. The titrations with anti H-2.8 and anti H-2.13 would seem to be an exception. In dilution 1:4, the maximal release for guinea pig complement equals that for rabbit complement (fig. 4). In these tests, however, only a one stage procedure was used for rabbit complement, whereas guinea pig complement was tested by a two stage procedure. Since both sera show marked anticomplementary

activity, the maximal release with rabbit complement can be expected to increase if the titration with rabbit complement were carried out in a two stage test, and should attain a value exceeding that for guinea pig complement.

Winn (1962) observed the same phenomenon of submaximal release when testing an allo-antiserum in the haemolytic technique against strain A red blood cells. In most instances he found no maximal haemolysis even when large amounts of guinea pig complement were used. It might be argued that this finding must be related to an amount of antibody insufficient to sensitize the cells, so that maximal lysis could not occur. But Winn clearly demonstrated that a large excess of antibody was present in his experiments.

The phenomenon of submaximal release with guinea pig complement might be due to anticomplementary factors, for these factors are known to affect guinea pig complement much more than rabbit complement (Winn 1965). However, in a two stage test in which the supernatant mouse sera were removed after incubation, thus eliminating anticomplementary factors, there still remained an unequivocal difference in maximal lysis between rabbit complement and guinea pig complement, the latter giving lower figures than the former (fig. 7). To exclude the possibility that this finding was related to the particular kind of assay used, the viability of sensitized cells after incubation with guinea pig complement was tested with the trypan blue dye exclusion method. We did not analyse this problem systematically, but in the few experiments performed the percentages of dead cells counted were the same as the percentages calculated from the ^{51}Cr release. This suggests that the finding of submaximal release is not confined to the ^{51}Cr assay.

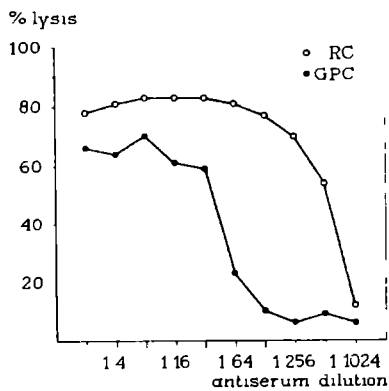


Figure 7: Effects of two complement preparations on the cytolysis of L1210 cells. Antiserum titrations with allo-antiserum H-2.31 (two stage test).

Unlike that obtained with allo-antisera, the maximal release obtained with the hetero-antiserum was the same with rabbit as with guinea pig complement. This is clearly demonstrated in fig. 6, which shows that in the titrations of the unabsorbed hetero-antiserum, only minor differences existed between the maximal lysis values for the two complements.

Discussion

The results of titrations with allo-antisera warrant the conclusion that guinea pig complement is inferior to rabbit complement in its ability to lyse sensitized mouse lymphoid cells. The titrations of H-2.8 and H-2.13 demonstrate that, with certain sera, no activity at all is in fact found. Moreover, the difference between the two complements extends to hetero-antisera. These comments of course apply only to the antisera we used. They were hyperimmune sera, all shown to be insensitive to 2-mercapto-ethanol. Such sera are believed to contain only IgG antibodies. As we pointed out, this is an important fact; for studies by Winn (1965) demonstrate that guinea pig complement can become even more efficient than rabbit complement if IgM antibodies are used as sensitizing agents.

There are several possible explanations of the decreased efficiency of guinea pig complement, namely:

- higher anticomplementary effects of mouse serum against guinea pig complement than against rabbit complement;
- decreased concentration of one or several of the complement components in guinea pig complement;
- a different mode of action of guinea pig complement.

Anticomplementary effects

Winn (1965) demonstrated that mouse serum can have a marked inhibitory effect on guinea pig complement. Sell (1964) has shown that the inhibition may be due to a complement-fixing antigen-antibody reaction between mouse serum and guinea pig serum. In our experiments, these factors played a decisive role when we tested the relatively weak antisera H-2.8 and H-2.13 (table IV and fig. 4). In both cases two stage tests showed an increase of activity with guinea pig complement. Even if anticomplementary effects were diminished by using a two stage test, however, the two complements continued to differ in efficiency. In the tests of the stronger sera (fig. 3), anticomplementary effects were not likely to influence the cytotoxic activity, for we demonstrated that these factors were of importance only in the first three or four dilutions of serum. The cytotoxic activity of these sera was calculated from the descending part of the titration curve

at higher dilutions. With antiserum H-2.31 we demonstrated, moreover, that the cytotoxic activity with both complements failed to rise in the two stage test. It is evident, therefore, that the difference in activity between the two types of complement cannot be explained on the basis of anticomplementary factors.

Decreased concentration of complement components

Evidently, a low concentration of a certain complement component might account for the decreased efficiency of guinea pig complement. However, Winn (1965) demonstrated that guinea pig complement can act very efficiently in a haemolytic system if cells are sensitized with IgM antibodies. It will be demonstrated in the next chapter, moreover, that guinea pig complement can become as active as rabbit complement in the presence of excess IgG antibody, as long as anticomplementary factors are ruled out. These findings strongly suggest that all components are intact in guinea pig complement.

Different mode of action

Haughton et al. (1969) suggested that the difference in efficiency between the two complements is probably due to the fact that, in the presence of IgG antibodies, guinea pig complement requires two reactive sites on the cell surface to be activated whereas rabbit complement needs only one. However, another requirement to be met before guinea pig complement can be activated is related to the geometry of the reactive sites. Even if there are many such sites, unless they are sufficiently close together on the cell surface, guinea pig complement is unable to act. In an antibody titration, cells are sensitized with decreasing amounts of antibody. At a certain antiserum dilution, a stage is reached in which not all antigenic sites on the cell are covered by the corresponding antibody; that is to say: there is no longer an antibody excess. If guinea pig complement requires twice as many sites as rabbit complement, the difference in efficiency is bound to become manifest as the antiserum is diluted further. It could be argued that this difference is not to be expected when hetero-antisera are used, because these contain many specificities and the concentration of reactive sites on the cell should therefore be higher than with monospecific antisera. This, however, is a misconception. One would expect to observe this phenomenon with all sera containing IgG antibodies, even if they are polyspecific. A serum containing many specificities likewise attains a dilution, in the titration, at which antibody excess no longer exists. Upon further dilution,

more and more antigenic sites remain uncovered, and a different activity of guinea pig complement is to be expected. Our experiments in fact confirmed this: a hetero-antiserum containing mouse-specific and strain-specific antibodies, showed the phenomenon just as monospecific allo-antisera did.

Although on theoretical grounds one might expect a lower efficiency of guinea pig complement with each antiserum used, as long as it contains no IgM antibodies, there may be quantitative variations which depend on the amount and characteristics of the specificities contained in the serum. The antigenic sites of two different specificities could be localized so close together that, when both are sensitized, an antigen-antibody complex forms which can activate guinea pig complement. A cell not lysed by sensitization with one specificity, will be destroyed if a mixture of the two specificities is used. As we discussed in the previous chapter, the literature on this point is confusing. Synergistic effects have been described both with guinea pig and with rabbit complement, whereas other studies have demonstrated a blocking effect with both types of complement.

For comparison of the separate sera with the antiserum mixtures in our tests, we calculated a hypothetical value which we call "the maximal expected cytotoxic activity". In view of the abovementioned findings, the use of this value calls for some elucidation.

If two sera are mixed in equal volumes, each separate serum is diluted twice: this means that the cytotoxic activity of each serum diminishes by a factor 2 if in the mixture either serum acts independently. The maximal cytotoxic activity attained in the mixture must therefore be the sum of the activities of the separate sera, divided by two. In this case the sera have a purely additive effect. Should there be synergistic effects, however, then the activity measured should be higher than the maximal expected value. Should it be lower, then this would mean some negative interference, possibly due to blocking effects.

The results of our experiments show a predominance of blocking effects in the tests with rabbit complement (table V). This would seem to warrant the conclusion that the antigenic sites involved are localized so close together that steric hindrance by antibody of different specifities plays a role. If this is true, one might expect synergistic effect with guinea pig complement; but no such effects were observed. In only one instance (4 + 3,28) was the value found just significantly higher than that calculated. An attempt to explain these conflicting results will be made in chapter 8.

Not only was it demonstrated that most allo-antibody mixtures showed no synergistic effects, but in a different approach it was made clear that the specificities present in a hetero-antiserum did not act synergistically either. The ratio RC/GPC, which is a measure of the relative efficiency of the two complements, did not change after absorption of the hetero-antiserum with (B10 x A)F_1 lymphoid cells. As mentioned, it was shown that hetero-antisera may contain H-2 specificities. These H-2 antibodies become apparent when mouse species specificities are removed by suitable absorption (Sachs et al. 1971). The hetero-antiserum left after absorption contained only antibody H-2.31. Guinea pig complement and rabbit complement had the same relative efficiency with this serum as with the unabsorbed antiserum.

The most plausible conclusion which emerges from these results is that the reactive sites of the specificities involved do not influence each other. For, if blocking influences were present with rabbit complement, the relative efficiency of this complement in the absorbed serum could be expected to increase. If synergistic effects were present in the titrations with guinea pig complement, on the other hand, the relative efficiency of this complement ought to diminish after absorption. Both phenomena should lead to an increased RC/GPC ratio in the absorbed serum. The absence of such an increase constitutes a strong argument in favour of the assumption that the antigenic sites in question are localized so far apart that a reciprocal influence is ruled out.

CHAPTER 7

COMPLEMENT TITRATIONS

Introduction

Complement has been studied most extensively in a standard haemolytic system in which two factors - cell surface antigens and antibody - are kept constant, the variable being complement which is tested in serial dilutions. For this purpose, use is made of a standard amount of sheep red blood cells sensitized with excess rabbit anti-sheep red cell antibody (amboceptor). After addition of complement the amount of lysis can be easily measured by the release of haemoglobin.

It has been shown that guinea pig complement can act very efficiently in this system, whereas rabbit complement is less efficient. We have mentioned studies by Winn (1965), who demonstrated that this difference is related to the molecular class of antibody used. Guinea pig complement is readily activated by antibodies of high molecular weight (IgM), while rabbit complement is more efficient when activated with antibodies of low molecular weight (IgG). The rabbit anti-sheep haemolysin commonly used in these tests, contains predominantly IgM antibodies (Paic 1939).

It seemed of interest to study these events in an allogeneic system, particularly because we have shown in the previous chapter that, in such a system, guinea pig complement is less effective than rabbit complement in the antibody titrations. In complement titration studies with mouse erythrocytes and allo-antisera, Winn (1965) demonstrated that guinea pig complement becomes at least as effective as rabbit complement if tested in antibody excess. However, when smaller amounts of antibody were used, rabbit complement was more efficient than guinea pig complement. These findings lend strong support to the theory that the number of reactive sites is of critical importance for the efficiency of guinea pig complement.

Haughton (1969) studied the lysis of mouse lymph-node cells sensitized with excess allo-antiserum and found that guinea pig complement was less efficient than rabbit complement. However, anticomplementary effects were not excluded in his assay because a one stage method was used (Haughton 1970).

In re-studying this problem, we have attempted to eliminate all factors impeding interpretation of results as best we could. To begin with, we used antisera containing only IgG antibodies. Secondly, all tests in which only slightly diluted antisera were used, were carried out in a two stage procedure in order to eliminate the anticomplementary effects of the sera.

Complement titrations

The two allo-antisera H-2.31 and H-2.3,28 were used in these titrations. They were tested separately and in a mixture of equal volumes. Moreover, both complements were tested after sensitization of the cells with excess hetero-antiserum. Whenever anticomplementary effects due to relatively undiluted wera could be expected, two stage procedures were used. An amount of cells (50 µl = 5 x 10^4cells) was sensitized during 15 minutes at 4°C with antibody (50 µl) of a given dilution, and centrifuged. The supernatants were removed and different dilutions of complement (50 µl) and Hank's BSS (100 µl) were added for incubation during 30 minutes at 37°C. Subsequent steps were the same as in the antiserum titrations. The results of each test were again plotted on log probit paper, and a line of best fit was drawn. One unit of complement was defined as the amount which lyses half the cells or, in other words, gives 50% lysis. This unit is therefore written C_{50}. The activity is expressed in units per ml. The following illustrates the actual calculation.

> Complement gives 50% lysis, say, at a dilution of 1/10. A 1/10 dilution therefore represents one C_{50} unit. The undiluted sample thus contains 10 C_{50} units. Since 50 µl complement is used in the test, the complement activity should be $\frac{10}{0.05}$ = 200 C_{50} units per ml.

The allo-antisera were first tested in two different dilutions: 1/16 and 1/64. A one stage method was used because in view of our experience with antibody titrations of these sera (fig. 3), no anticomplementary effects were expected at these dilutions. The results of these titrations are presented in fig. 8. The complement activity was calculated from these curves (table VII). It is evident that guinea pig complement was less active than rabbit complement at a serum dilution of 1/16.

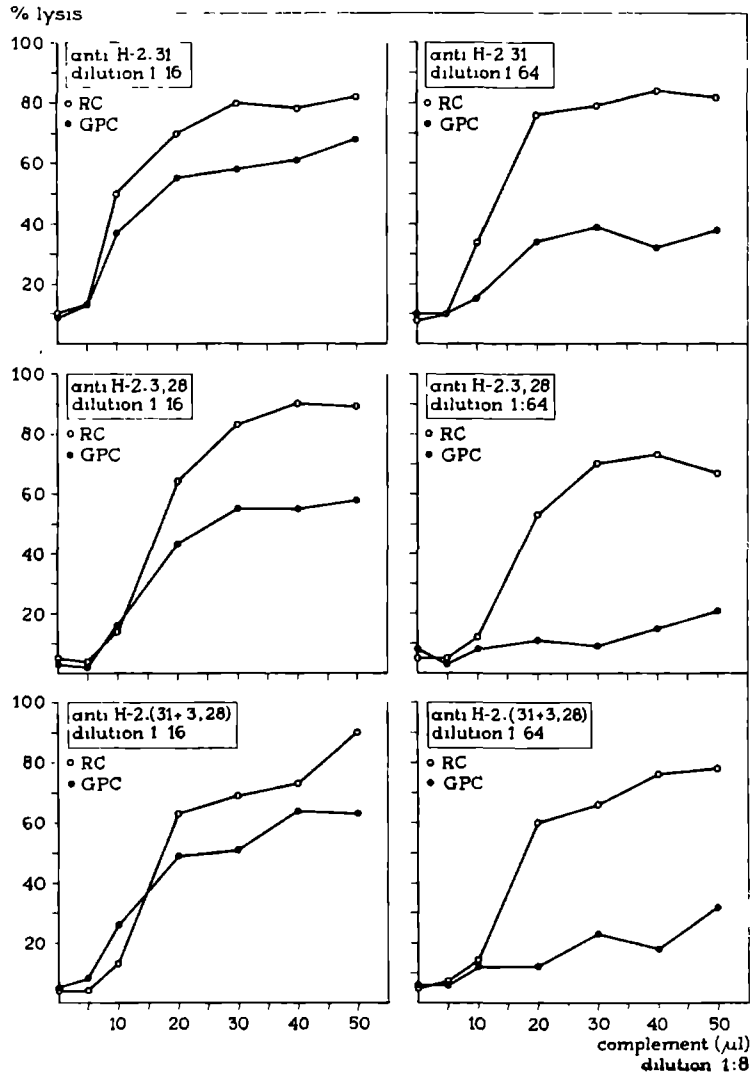


Figure 8: Effects of two complement preparations on the cytolysis of L1210 cells. Complement titrations with allo-antisera.

At the higher dilution (1/64), the activity of rabbit complement remained unchanged whereas that of guinea pig complement diminished to very low levels. An unmistakable diminution of the efficiency of guinea pig complement thus occurred when a limited antibody system was used. It seemed of interest to establish whether the

TABLE VII: EFFECTS OF TWO COMPLEMENT PREPARATIONS ON THE CYTOLYSIS OF L1210 CELLS. COMPLEMENT ACTIVITIES AFTER SENSITIZATION WITH ALLO-ANTISERA AND WITH AN ANTISERUM MIXTURE

Specificities	Antiserum dilution	Complement activity	
		Rabbit C (C_{50} U/ml)	Guinea Pig C (C_{50} U/ml)
31	1/16	640	400
	1/64	620	<160
3,28	1/16	440	340
	1/64	420	<160
31 + 3,28	1/16	430	340
	1/64	400	<160

activity of guinea pig complement would increase to a level comparable with that of rabbit complement if larger amounts of antibody were used to sensitize the cells. One of the antisera (H-2.31) was therefore used in a 1/4 dilution. A two stage procedure was used this time because this serum could be expected to show marked anticomplementary effects. The outcome of this titration is shown in fig. 9, which reveals that the maximal release remained below that with rabbit complement.

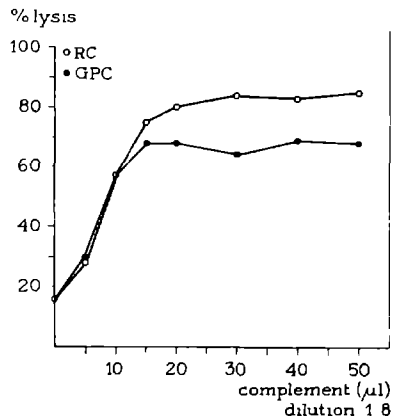


Figure 9: Effects of two complement preparations on the cytolysis of L1210 cells. Complement titrations with excess antibody H-2.31 (dilution 1:4) (two stage test).

This finding is in agreement with the results of the antibody titrations, which likewise disclosed a submaximal release with guinea pig complement. At lower complement concentrations, however, the efficiency of guinea pig complement equalled that of rabbit complement. In no case was guinea pig complement found to be more efficient than rabbit complement (as it is in the haemolytic system with sheep red cells and rabbit antibody).

It has been shown in the chapter on antibody titrations that a hetero-antiserum behaved in the same way as the allo-antisera. At higher antibody dilutions, guinea pig complement was less efficient than rabbit complement (fig. 6). The only difference from the titrations of allo-antisera was that with the hetero-antiserum the same maximal lysis was achieved for both complements.

As we mentioned before, guinea pig complement is much more efficient than rabbit complement if tested with excess hetero-antiserum containing mainly IgM antibodies, i.e. in the haemolytic system with sheep red cells and rabbit antibody. What if guinea pig complement were tested in a similar system but with excess antiserum containing only IgG antibodies? The result of such a titration is presented in fig. 10. Unabsorbed rat anti-B10.D2 serum was used as sensitizing agent.

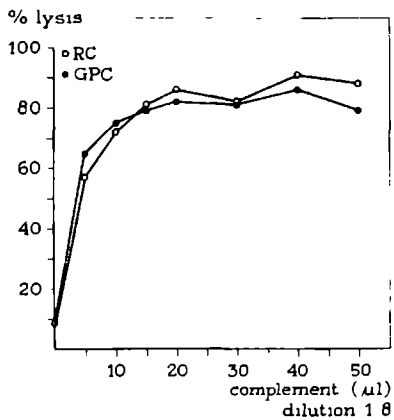


Figure 10: Effects of two complement preparations on the cytolysis of L1210 cells. Complement titrations with excess hetero-antiserum (rat anti B10.D2 serum, dilution 1:16) (two stage test).

The 1/16 dilution supplied excess antibody. Both complements had a similar activity, and in this case guinea pig complement gave a maximal release as predicted from our findings in the antibody titration study.

Discussion

The results of the complement titration study furnish additional evidence that it is not a decrease in the concentration of complement components that causes the lower efficiency of guinea pig complement. The efficiency is much more likely to be correlated with the concentration of reactive sites on the cell surface. The two complements are equally efficient in antibody excess. In a limited antibody system (1/64 dilution), the activity of guinea pig complement is already diminished to very low levels while that of rabbit complement is still unchanged.

Our tests with allo-antisera gave results which were in contrast to the findings reported by Haughton et al. (1969). They showed that guinea pig complement was about three times less efficient than rabbit complement when tested with excess allo-antibody. In our preliminary experiments we found that mouse serum can have marked inhibitory effects on these reactions. The lower activity found by Haughton was most likely caused by anticomplementary effects because he did not use the two stage procedure.

Some of the results described by Winn (1965) suggest that guinea pig complement can become even more efficient than rabbit complement if tested with excess allo-antibody and mouse erythrocytes. In our study, in which we used antisera containing only IgG, we never observed a higher efficiency of guinea pig complement. Perhaps Winn's serum contained small amounts of IgM antibodies, which might be responsible for his findings. We were also unable to demonstrate a higher activity of guinea pig complement when we tested a hetero-antiserum also containing no IgM antibody. This supports Winn's assumption that the high efficiency of guinea pig complement in the haemolytic system may be ascribed to the predominance of IgM antibodies in this system.

CHAPTER 8

CONCLUSIONS

The results of our study tend to corroborate reports by other investigators (Boyse et al. 1962, Winn 1965, Haughton et al. 1969) stating that there can be substantial differences in complement activity between sera from different species. This is of importance inasmuch as we have demonstrated that it can sometimes lead to false negative results in the cytotoxic test. It is probable that, to be activated, guinea pig complement requires two reactive sites of IgG. Why this is so has not been established, but the best explanation so far seems to be that it must be related to some property of that complement component that actually binds with the antibody, i.e. C1q. The actual mechanism of action, however, remains obscure.

In our experiments with allo-antiserum mixtures we found blocking effects of H-2 specificities in the presence of rabbit complement (table V). This suggests that the antigenic determinants involved are localized very close together on the cell membrane. In view of these findings, we expected to be able to demonstrate synergism between H-2 specificities with guinea pig complement. This, however, proved generally not to be the case. The best explanation for these apparently conflicting results would seem to be that, with guinea pig complement, synergism as well as inhibition occurs. This is elucidated in fig. 11, in which antigen a and antigen b are localized so close together that competition can occur in the binding of antibodies of specificity a and specificity b. This means that, with antibody mixtures and rabbit complement, blocking effects are found. Assuming guinea pig complement to be capable of bridging a distance of, say, four reactive sites, the course of the activation can be conceived of as follows. Upon addition of only antibody a, two reactive sites occur which are sufficiently close together to activate guinea pig complement (instance A). The same applies

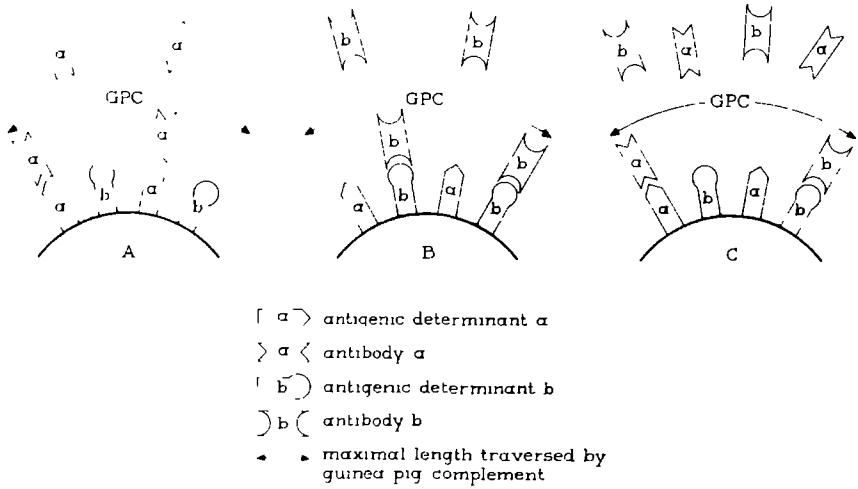


Figure 11: Schematic representation of the action of guinea pig complement in the presence of monospecific antisera separately (A and B) and of an antiserum mixture (C).

to antibody b (instance B). Upon addition of a mixture of a and b antibodies, two of the four available antigenic sites are not sensitized due to steric inhibition. Guinea pig complement will nevertheless be activated because it is still just capable of bridging the distance between the two farther spaced reactive sites (instance C). The ultimate result will be that no difference occurs between activation with the separate antisera and that with the mixture. This means that only an additive effect will be observed. But this applies only of the two antigenic determinants a and b are available in equal concentration. If the antigenic determinants are present on the cell membrane in unequal concentrations, it is quite conceivable that synergistic or blocking effects can nevertheless sometimes be predominant in the presence of guinea pig complement.

The above conception is probably an extreme simplification of the true events. But it affords a plausible explanation of the results observed, which can be summarized as follows. In the presence of rabbit complement, blocking effects are invariably observed if the antigenic sites are sufficiently close together to cause steric hindrance. If the same specificities are tested with guinea pig complement, one may expect additive, synergistic or blocking effects, dependent on the concentration of the various determinants. Conclusions on the spacing of the various determinants may therefore be drawn only from the results

obtained with rabbit complement. Since we observed blocking effects in nearly all instances with rabbit complement, we must assume that the H-2 antigens studied are localized close together on the cell membrane.

Boyse (1968) demonstrated that an H-2 antibody which reacts specifically with a D end antigen, can inhibit the absorption of an anti D end antibody of different specificity (blocking effect). However, the absorption of an anti K end antibody was not influenced by an already absorbed anti D end antibody. He concluded from these facts that D end and K end antigens are not localized close together on the cell membrane. Our results fail to confirm this. In a combination of antibody H-2.31 (anti K end) and antibody H-2.4 (anti D end), an unmistakable blocking effect was observed (table V). It is difficult to form any conclusion concerning the blocking effect of anti H-2.31 and anti H-2.3,28, because there are indications that antigen 3 can be present as a K end as well as a D end antigen (Shreffler 1971).

On the other hand, the absence of blocking or synergism in the titration with hetero-antiserum (table VI) suggests that H-2 antigens and mouse species antigens are localized farther apart. This implies that the antigens are not evenly distributed over the cell surface but occur in "clusters". Winn inferred the same hypothesis from his studies of complement fixation on mouse lymph-node and thymus cells (Winn 1962). The hypothesis is also quite consistent with the immunohistochemical studies of Cerottini and Brunner (1967), who demonstrated by means of a semi-quantitative immunofluorescent technique that H-2 antigens are localized in discrete areas on the cell surface.

We must therefore assume that clusters of allo-antigens and clusters of mouse species antigens occur. Moreover, the fact that blocking effects occur between the allo-antisera tested warrants the conclusion that the allo-antigenic clusters are made up of antigens of different specificity. Finally, our observations failed to show that D end and K end antigens occur in separate clusters, as Boyse et al. (1968) suggested. We could demonstrate blocking effects between these antigens. This observation shows that D end and K end antigens can occur in the same cluster of antigenic determinants.

A submaximal release of ^{51}Cr in the presence of guinea pig complement and excess antibody was found in all titrations with allo-antisera (figs. 3 and 5). The unabsorbed hetero-antiserum, however, showed a virtually equal maximal release with both types of complement (fig. 6). Similar effects were observed in the complement titrations with allo-antisera and hetero-antiserum (figs. 8, 9 and 10). Since these findings cannot be explained by the influence of anticomplementary effects, it seems a plausible spe-

culation that it might be related to the concentration of reactive sites on the cell surface. Studies by Winn (1962) demonstrated that the concentration of an antigenic specificity on the cell surface is not the same for each cell within a given population. Some cells show a high concentration of antigenic determinants, whereas other cells have only a few reactive sites on their surfaces. Assuming that this also applies to L1210 cells, especially as these cells have not been synchronized and are at different stages of division, we may expect the various concentrations to be distributed in accordance with a β distribution* between minimum and maximum concentration. This situation is shown in fig. 12.

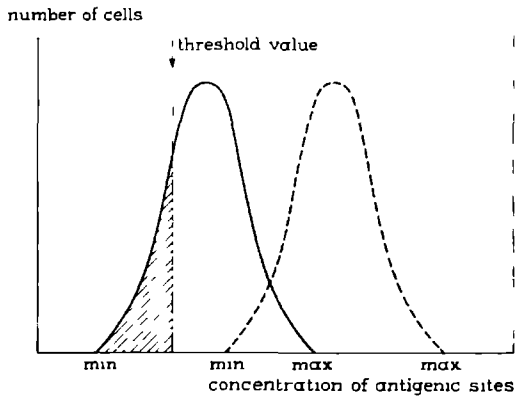


Figure 12: Hypothetical β-distribution of antigenic determinants within a cell population, directed against one H-2 specificty (———) or against multiple specificities of the hetero-antiserum (------) Cells within shaded area have too low a concentration of antigenic sites and will be completely resistant to lysis in the presence of guinea pig complement.
min. = minimum concentration.
max. = maximum concentration.

Sensitization with an excess of a monospecific antiserum might lead to such a low concentration of reactive sites on some cell surfaces that guinea pig complement cannot be activated, whereas rabbit complement is still able to lyse these cells because it

* The β distribution for concentration c between its minimum (min) and its maximum (max) is characterized by the formula: constant x $(c - min)^{\alpha}$ $(max - c)^{\beta}$,

requires only half the reactive sites needed by guinea pig complement. A certain percentage of the cells will therefore be resistant to guinea pig complement so that lysis is submaximal. But when the cells are sensitized with excess antiserum containing many specificities (e.g. a hetero-antiserum), the concentration of reactive sites on all cells will be sufficiently high to permit of their lysis by guinea pig complement. This hypothesis can explain our findings on maximal release (figs. 3 and 5). We made no study of the effects of larger amounts of complement in this situation, but the aforementioned experiments by Winn suggest that, even with large amounts of complement, some cells will remain resistant to lysis (Winn 1962).

The role of complement in graft rejection has not been elucidated. Some reports in the literature suggest that complement can be as active in cell destruction as it is in cytotoxic assays in vitro. When cells are incubated with specific antibody in vitro, they remain viable until complement is added. But when cells sensitized with antibody are injected into compatible animals, they are rapidly destroyed. Winn (1960b) demonstrated this with inoculates of 6C3HED lymphoma cells sensitized with C57Bl/6 anti 6C3HED serum. The tumours failed to grow in C3H mice unless large amounts of cells were used. Winn concluded that the isogeneic host provided the complement to destroy the sensitized cells. In addition he demonstrated that, even with inoculates of large numbers of cells, growth could be prevented by pre-treating the animals with guinea pig complement. At least in these studies, the in vivo effect of complement was comparable to its in vitro activity.

There are several reports on the role of complement in the rejection of kidney grafts. Gewurz, Clark, Finstad, Kelly, Varco, Good and Gabrieson (1966) found a slight reduction of complement titre during renal graft rejection in dogs. Guiney, Austen and Russell (1964) demonstrated a pronounced diminution of the titre of the second complement component during renal allograft rejection in man. The whole complement titre did not significantly change in their study. The titre of the second component seems to be a more sensitive index of complement fixation in these cases, and this can be explained from our knowledge of complement activation. The decay of SAC $\overline{1,4,2}$ to stage SAC $\overline{1,4}$ is very rapid at 37°C. New C2 molecules are used to rebuild a SAC $\overline{1,4,2}$ complex, and in this cyclic process C2 is likely to be consumed during activation. The C2 titre consequently diminishes much more than the titres of the other C components or the whole complement titre. Radial immunodiffusion studies by Carpenter, Gell, Merrill and Dammin (1967) have demonstrated that the third

complement component also decreases during rejection in man. In a later study, moreover, investigators of this group demonstrated hypercatabolism of C3 and C4 in patients with renal allograft rejection (Carpenter, Ruddy, Shehadeh, Müller-Eberhard, Merrill and Austen 1969).

All these findings suggest that complement can play a role in graft rejection. On the other hand, however, it has been demonstrated that mouse strains deficient in the fifth complement component reject skin allografts exactly as do mice without this deficiency (Caren and Rosenberg 1965). It seems possible, therefore, that not all complement components are required to destroy foreign cells in vivo.

Thus, although there are unmistakable indications of in vivo processes comparable to in vitro events, it is by no means certain that results of in vitro studies can in fact be applied unchanged to the transplantation reaction.

Nevertheless it seems probable that in vitro cytotoxic assays can give an understanding of the mechanism of the immune response. This applies in particular to the study of immunological enhancement. As we mentioned, it is a prerequisite for achieving enhancement that the antisera used contain antibodies directed against all strong transplantation antigens which the graft introduces as foreign into the host. Otherwise, the uncovered antigens could lead to graft rejection. By designing a model in which the donor possesses two or three strong transplantation antigens which the host does not possess, an attempt can be made to achieve an enhancing effect by immunizing the host with a mixture of serum containing antibodies directed against all these specificities. However, if there were synergistic effects between these specificities in terms of cytolysis, then the cytolytic effect on the graft in vivo could be expected to increase; and no enhancement would occur. Our in vitro results with the antiserum suggest no such synergism in the majority of instances. Our findings therefore warrant the expectation that enhancement can be achieved in vivo when serum mixtures are used in models comprising more than one antigenic difference. While clinical application of these principles may still seem far away, the results of studies of this kind are likely eventually to provide the tools for improved treatment of graft rejection in man.

Summary

This study compares the activities of rabbit complement and guinea pig complement in terms of their ability to lyse L1210 tumour cells sensitized with antibody. Cytolysis was demonstrated by means of a ^{51}Cr release assay. Optimally monospecific H-2 allo-antisera and a rat anti-mouse lymphocyte serum were used as antibody.

1. In the antiserum titrations, guinea pig complement was invariably less efficient than rabbit complement, in the titrations with allo-antisera as well as in those with the hetero-antiserum.
2. The mouse sera used proved to contain strong anticomplementary factors, which were most evident in the titrations with guinea pig complement. In some instances this even led to false negative results. However, the difference in efficiency between the two types of complement was not explained by these factors, for after their elimination the guinea pig complement remained less active than rabbit complement.
3. Data from the literature as well as the results of our own complement titration study make it unlikely that the difference in activity might be explained by a decreased concentration of some complement component of guinea pig serum. The most plausible explanation would seem to be that, to be activated, guinea pig complement requires more reactive sites on the cell surface than rabbit complement. However, this applies only if IgG antibodies are used as sensitizing agents.
4. When mixtures of monospecific H-2 antisera were titrated, blocking effects were found to occur in the presence of rabbit complement. This phenomenon indicates steric hindrance of antibody binding, and suggests that the antigenic determinants involved are localized very close together on the cell membrane. On theoretical grounds, synergistic effects might be expected with guinea pig complement in titrations of combinations of allo-antisera. In most experiments, however, no such effects were observed. These seemingly conflicting results might be explained by the assumption that synergistic as well as inhibitory effects play a role in the titrations with guinea pig complement.
5. Neither synergistic nor blocking effects were demonstrable between the species-specific and the strain-specific antibodies present in the hetero-antiserum. This would seem to suggest that the distance between species-specific and strain-specific antigenic sites on the cell membrane is so large that

a reciprocal influence is ruled out.

6. The results obtained warrant the conclusion that the antigenic determinants lie on the cell surface in "clusters". The clusters of allo-antigens are made up of determinants of different H-2 specificity. Moreover, the D end and K end antigens studied were found to occur together in the same clusters.
7. All titrations in which allo-antisera were used with guinea pig complement, were characterized by submaximal release of ^{51}Cr. This phenomenon is probably due to the fact that a proportion of the cell population is resistant to lysis as a result of an insufficient concentration of antigenic determinants. Cells sensitized with a hetero-antiserum, however, have such a density of reactive sites on the surface that the phenomenon of submaximal release is not observed.
8. When excess antibody was used in the complement titrations, the activity of guinea pig complement and that of rabbit complement were on a comparable level. In a limited antibody system, the activity of guinea pig complement was significantly diminished while that of rabbit complement remained unchanged. This is another argument in favour of the theory which explains the difference found between the two types of complement on the basis of the concentration of antigenic sites.
9. The principal application of the results of this study in clinical transplantation is to be found in the phenomenon of immunological enhancement. In principle, it would seem to be possible to use mixtures of antisera in order to induce enhancement without increasing the risk of cytolysis, and therefore of graft rejection, by synergistic effects of the antibodies used.

Samenvatting

In deze studie werden de activiteiten van konijnecomplement en caviacomplement vergeleken met betrekking tot hun vermogen om met antilichaam gesensitizeerde L1210 tumorcellen te lyseren. De cytolysis werd aangetoond met behulp van een ^{51}Cr release assay. Als antilichaam werden zo goed mogelijk monospecifieke H-2 alloantisera en een rat anti-muis lymphocytenserum gebruikt.

1. In de antiserum titraties bleek caviacomplement steeds minder effectief dan konijnecomplement. Dit gold zowel voor de titraties met de allo-antisera, als voor die met het hetero-antiserum.
2. De gebruikte muizensera bleken sterke anticomplementaire factoren te bevatten, die vooral in de titraties met caviacomplement optraden. Dit leidde zelfs in enkele gevallen tot vals negatieve resultaten. Desondanks kon het verschil in effectiviteit tussen beide complementen hierdoor niet verklaard worden. Immers, ook na eliminatie van de anticomplementaire invloeden, bleef caviacomplement minder actief dan konijnecomplement.
3. Zowel de literatuurgegevens als onze eigen resultaten met de complement titraties maken het onwaarschijnlijk dat het verschil in activiteit verklaard kan worden uit een verminderde concentratie van een der componenten van caviacomplement. De meest plausibele verklaring is dat caviacomplement meer antigeen-antilichaam complexen nodig heeft dan konijnecomplement om gesensitizeerd te worden. Dit geldt echter alleen, als IgG bevattende antisera gebruikt worden.
4. Indien mengsels van mono-specifieke H-2 antisera getitreerd werden, bleken er in de aanwezigheid van konijnecomplement blokkerende effecten op te treden. Dit fenomeen wijst op sterische inhibitie van de antilichaambinding en suggereert dat de betrokken antigene determinanten zeer dicht bijeen liggen op de celmembraan. Op theoretische gronden zou men met caviacomplement synergistische effecten kunnen verwachten, in titraties met combinaties van allo-antisera. Deze werden echter in de meeste experimenten niet waargenomen. Deze schijnbaar tegenstrijdige resultaten zou men kunnen verklaren door aan te nemen dat in de titraties met caviacomplement zowel synergistische als blokkerende effecten een rol spelen.
5. Er konden geen synergistische noch blokkerende effecten worden aangetoond tussen de species-specifieke en stam-specifieke antilichamen, die aanwezig waren in het hetero-antiserum. Dit pleit ervoor dat de afstand tussen species-specifieke en stamspecifieke antigenen op de celmembraan zó groot is, dat zij elkaar onderling niet kunnen beïnvloeden.

6. Uit de gevonden resultaten kan worden geconcludeerd dat de antigene determinanten in "trosjes" op het celoppervlak bijeen liggen. De trosjes van allo-antigenen zijn opgebouwd uit determinanten van verschillende H-2 specificiteit. Bovendien is gebleken dat de onderzochte D end en K end antigenen samen in dezelfde trosjes voorkomen.
7. In alle titraties, waarin allo-antisera tezamen met caviacomplement gebruikt werden, ontbrak een maximale release van ^{51}Cr. Dit verschijnsel wordt waarschijnlijk veroorzaakt doordat een deel van de celpopulatie resistent is tegen lysis als gevolg van een te lage concentratie van antigene determinanten. Als de cel daarentegen gesensitizeerd is met een heteroantiserum, dan is deze zo dicht bezet met antigeen-antilichaam complexen, dat er wel een maximale release optreedt.
8. Indien in de complement titraties overmaat antilichaam werd gebruikt, dan was de activiteit van caviacomplement vergelijkbaar met die van konijnecomplement. In een systeem met beperkte hoeveelheid antilichaam was er een significante daling van de activiteit van caviacomplement, terwijl de activiteit van konijnecomplement onveranderd bleef. Dit pleit opnieuw voor het belang van de concentratie van antigene determinanten ter verklaring van de gevonden verschillen tussen beide complementen.
9. De belangrijkste toepassing van deze studie voor de klinische transplantatie wordt gevonden in het verschijnsel immunologische enhancement. Het lijkt in principe mogelijk om mengsels van antisera te gebruiken voor het induceren van enhancement, zonder dat door synergistische effecten van de gebruikte antilichamen de kans op cellysis, en dus afstoting van het transplantaat, vergroot wordt.

References

Amos, B., Koprowsky, H. (1963) Cell bound antibodies: Conference of the National Academy of Sciences - National Research Council, May 10, 1963. Philadelphia, Wistar Institute Press.

Andersson, B., Wigzell, H., Klein, G. (1967) Some characteristics of 19S and 7S mouse isoantibodies in vivo and in vitro. Transplantation 5, 11.

Audran, R. (1970) Notions actuelles concernant le complément. Rev. Eur. Etud. Clin. Biol. 15, 610.

Borsos, T., Dourmashkin, R.R., Humphrey, J.H. (1964) Lesions in erythrocyte membranes caused by immune haemolysis. Nature 202, 251.

Borsos, T., Rapp, H.J. (1965) Complement fixation on cell surfaces by 19S and 7S antibodies. Science. 150, 505.

Boyle, W. (1968) An extension of the ^{51}Cr-release assay for the estimation of mouse cytotoxins. Transplantation 6, 761.

Boyse, E.A., Old, L.J., Thomas, G. (1962) A report on some observations with a simplified cytotoxic test. Transplant. Bull. 29, 63.

Boyse, E.A., Old, L.J., Stockert, E. (1968) An approach to the mapping of antigens on the cell surface. Proc. Natl. Acad. Sci. U.S.A. 60, 886.

Caren, L.D., Rosenberg, L.T. (1965) Complement in skin grafting in mice. Immunology 9, 359.

Carpenter, C.B., Gill, T.J., Merrill, J.P., Dammin, G.J. (1967) Alterations of human serum β_{1C}-globulin ($C\bar{3}$) in renal transplantation. Am. J. Med. 43, 854.

Carpenter, C.B., Ruddy, S., Shehadeh, I.H., Müller-Eberhard, H.J., Merrill, J.P., Austen, K.F. (1969) Complement metabolism in man: Hypercatabolism of the fourth (C4) and third (C3) components in patients with renal allograft rejection and hereditary angioedema (HAE). J. Clin. Invest. 48, 1495.

Cerottini, J.C., Brunner, K.T. (1967) Localisation of mouse isoantigens on the cell surface as revealed by immunofluorescence. Immunology 13, 395.

Chambers, R., Fell, H.B. (1931) Micro-operations on cells in tissue cultures. Proc. R. Soc. Lond. (Biol.), 109, 380.

Cresswell, P., Sanderson, A.R. (1968) Spatial arrangement of H-2 specificities: evidence from antibody adsorption and kinetic studies. Transplantation 6, 996.

Davies, D.A.L. (1969) The molecular individuality of different mouse H-2 histocompatibility specificities determined by single genotypes. Transplantation 8, 51.

Foley, E.J. (1953) Antigenic properties of methylcholanthrene induced tumors in mice of strain of origin. Cancer Res. 13, 835.

Frank, M.M., Gaither, T. (1970a) The effect of temperature on the reactivity of guinea pig complement with γG and γM haemolytic antibodies. Immunology 19, 967.

Frank, M.M., Gaither, T. (1970b) Evidence that rabbit γG haemolysin is capable of utilizing guinea pig complement more efficiently than rabbit γM haemolysin. Immunology 19, 975.

Freund, J. (1951) The effect of paraffin oil and mycobacteria on antibody formation and sensitization. Am. J. Clin. Pathol. 21, 645.

Gewurz, H., Clark, D.S., Finstad, J., Kelly, W.D., Varco, R.L., Good, R.A., Gabrielsen, A.F. (1966) The role of complement system in graft rejections in experimental animals and man. Ann. N.Y. Acad. Sci. 129, 673.

Goodman, H.S. (1961) A general method for the quantitation of immune cytolysis. Nature 190, 269.

Gorer, P.A. (1937) The genetic and antigenic basis of tumour transplantation. J. Pathol. Bacteriol. 44, 691.

Gorer, P.A., O'Gorman, P. (1956) The cytotoxic activity of isoantibodies in mice. Transplant. Bull. 3, 142.

Graff, R.J., Silvers, W.K., Billingham, R.E., Hildemann, W.H., Snell, G.D. (1966) The cumulative effect of histocompatibility antigens. Transplantation 4, 605.

Guiney, E.J., Austen, K.F., Russell, P.S. (1964) Measurement of serum complement during homograft rejection in man and rat. Proc. Soc. Exp. Biol. Med. 115, 1113.

Hanks, J.H., Wallace, R.E. (1949) Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exp. Biol. Med. 71, 196.

Haughton, G., Mc Gehee, P.M. (1969) Cytolysis of mouse lymphoid cells by allo-antibody: a comparison of guinea pig and rabbit complements. Immunology 16, 447.

Haughton, G. (1970) Personal communication.

Holmes, E.C., Morton, D.L., Schidlovsky, G., Trahan, E. (1971) Cross-reacting tumor-specific transplantation antigens in methylcholanthrene-induced guinea pig sarcomas. J. Natl. Cancer Inst. 46, 693.

Humphrey, J.H., Dourmashkin, R.R. (1965) Electron microscope studies of immune cell lysis. Ciba Foundation Symposium on complement p. 175. London, Churchill.

Ivasková, E., Vybíralová, H., Raue, I., Démant, P., Iványi, P. (1969) Synergic action of HL-A antibodies. Folia Biol. (Praha), 15, 26.

Kaliss, N. (1969) Immunological enhancement. Int. Rev. Exp. Pathol. 8, 241.

Klein, G., Klein, E. (1962) Antigenic properties of other experimental tumors. Cold Spring Harbor Symposium Quant. Biol. 27, 643.

Koene, R., McKenzie, I.F.C., Painter, E., Sachs, D.H., Winn, H.J., Russell, P.S. (1971) Soluble mouse histocompatibility antigens. Transplant. Proc. 3, 231.

Kristofová, H., Lengerová, A., Rejzková, J. (1970) Indirect mapping of spatial distribution of some H-2 antigens on the cell membrane. Folia Biol. (Praha) 16, 81.

Levy, R.L., Lepow, I.H. (1959) Assay and properties of a serum inhibition of C'1-esterase. Proc. Soc. Exp. Biol. Med. 101, 608.

Lachmann, P.J. (1968) Complement. in: P.G.H. Gell and R.R.A. Coombs: Clinical aspects of immunology. 2nd ed. Oxford, Blackwell, p. 384.

Law, L.W., Dunn, T.B., Boyle, P.J., Miller, J.H. (1949) Observations on the effects of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Natl. Cancer Inst. 10, 179.

Mayer, M.M. (1961) Complement and complement fixation. in: E.A. Kabat and M.M. Mayer: Experimental Immunochemistry, Springfield. C.C. Thomas, 2nd ed.

Mayer, M.M. (1965) Mechanism of hemolysis by complement. in: Ciba Foundation Symposium on Complement. London, Churchill. p. 4.

McKenzie, I., Jeekel, J., Koene, R., Winn, H.J. (1970) Enhancement for skin grafts in the mouse. Fed. Proc. 29, 3049. (abstract)

McKenzie, I.F.C., Koene, R., Winn, H.J. (1971) Mechanism of skin graft enhancement in the mouse. Transplant. Proc. 3, 711.

Middlebrook, G.(1950) A hemolytic modification of the hemagglutination test for antibodies against tubercle bacillus antigens. J. Clin. Invest. 29, 1480.

Möller, E., Möller, G. (1962) Quantitative studies of the sensitivity of normal and neoplastic mouse cells to the cytotoxic action of isoantibodies. J. Exp. Med. 115, 527.

Möller, E., Eklund, A.E. (1965) Cytotoxic effects of iso-antibodies directed against ABO and Rh antigens on human lymph node cells. Nature 206, 731.

Möller, G. (1961) Demonstration of mouse isoantigens at the cellular level by the fluorescent antibody technique. J. Exp. Med. 114, 415.

Morgan, J.F., Morton, H.J., Parker, R.C. (1950) Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exp. Biol. Med. 73, 1.

Müller-Eberhard, H.J., Polley, M.J., Nilsson, U.R. (1966) Molecular events during immune cytolysis in immunopathology. in: IVth Int. Symposium, Monte Carlo 1965. Basel, Schwabe,p. 421.

Munoz, J. (1957) Production in mice of large volumes of ascites fluid containing antibodies. Proc. Soc. Exp. Biol. Med. 95, 757.

Muschel, L.H. (1965) Immune bactericidal and bacteriolytic reactions. in: Ciba Foundation Symposium on Complement. London, Churchill, p. 155.

Paic, M. (1939) Ultracentrifugation de l'hémolysine. Détermination de sa constante de sédimentation et de son poids moléculaire. Bull. Soc. Chim. Biol. 21, 412.

Pappenheimer, A.M. (1917) Reactions of lymphocytes under experimental conditions. J. Exp. Med. 25, 633.

Pondman, K.W. (1969) Het complement-systeem. Ned. Tijdschr. Geneeskd. 113, 1473.

Ross, A., Lepow, I.H. (1960) Studies on immune cellular injury. I. The cytotoxic effects of antibody and complement. J. Exp. Med. 112, 1085.

Rosse, W.F., Parker, J. (1968) Fixation of the first component of complement (C'1a) by human antibodies. J. Clin. Invest. 47, 2430.

Sachs, L., Feldman, M. (1958) Cytotoxic antibodies in the homograft reaction. J. Natl. Cancer Inst. 21, 563.

Sachs, D.H., Winn, H.J., Russell, P.S. (1970) Detection of H-2 specificities across a species barrier. Symposium on Immunogenetics of the H-2 system, Prague, Sept. 1-4.

Sachs, D.H., Winn, H.J., Russell, P.S. (1971) Histocompatibility relationship between species. Transplant. Proc. 3, 210.

Sanderson, A.R. (1964) Cytotoxic reactions of mouse iso-antisera: preliminary considerations. Brit. J. Exp. Pathol. 45, 398.

Sell, K.W. (1964) in: Ciba Foundation Symposium on Complement (1965) London, Churchill, p. 149.

Shreffler, D.C., Snell, G.D. (1969) The distribution of thirteen H-2 alloantigenic specificities among the products of eighteen H-2 alleles. Transplantation 8, 435.

Shreffler, D.C. (1970) in: D. Aminof (ed) Blood and tissue antigens, New York, Academic Press, p. 85.

Shreffler, D.C., David, C.S., Passmore, H.C., Klein, J. (1971) Genetic organization and evolution of the mouse H-2 region. A duplication model. Transplant. Proc. 3, 176.

Snell, G.D. (1948) Methods for the study of histocompatibility genes. Genetics 49, 87.

Snell, G.D. (1958) Histocompatibility genes of the mouse. II. Production and analysis of isogenic resistant lines. J. Natl. Cancer Inst. 21, 843.

Snell, G.D., Hoecker, G., Amos, D.B., Stimpfling, J.H. (1964) A revised nomenclature for the histocompatibility-2 locus

of the mouse. Transplantation 2, 777.
Snell, G.D. Catalog of mouse alloantisera 1968.
Uhr, J.W., Finkelstein, M.S. (1963) Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage $\phi\chi$ 174. J. Exp. Med. 117, 457.
Wigzell, H. (1965) Quantitative titrations of mouse H-2 antibodies using Cr^{51}-labelled target cells. Transplantation 3, 423.
Winn, H.J. (1960a) The immune response and the homograft reaction. Natl. Cancer Inst. Monogr. 2, 113.
Winn, H.J. (1960b) Immune mechanisms in homotransplantation. I. The role of serum antibody and complement in the neutralization of lymphoma cells. J. Immunol. 84, 530.
Winn, H.J. (1962) The participation of complement in iso-immune reactions. Ann. N.Y. Acad. Sci. 101, 23.
Winn, H.J. (1965) Effects of complement on sensitized nucleated cells. Ciba Foundation Symposium on Complement. London, Churchill, p. 133.
Winn, H.J. (1970) Humoral antibody in allograft reactions. Transplant. Proc. 2, 83.

S T E L L I N G E N

1. Een vergelijking tussen de werking van cavia- en konijnecomplement is alleen mogelijk, als er rekening wordt gehouden met de klasse van de antilichamen die gebruikt worden.

   Dit proefschrift.

2. Bij het opsporen van circulerende antilichamen in het serum van een potentiële ontvanger van een transplantaat door middel van een directe kruisproef is het gebruik van een "two stage" procedure onontbeerlijk.

   Dit proefschrift.

3. Immunologische enhancement wordt niet veroorzaakt door een efferente blokkade van de immuunresponse.

   McKenzie, I.F.C., Koene, R., Winn, H.J. (1971) The mechanism of skin graft enhancement in the mouse. Transplant. Proc. 3, 711.

4. Het verdient overweging een nefrotisch syndroom door "minimal lesions" in eerste instantie te behandelen met cyclofosfamide.

   Moncrieff, M.W., White, R.H.R., Ogg, C.S., Cameron, J.S. (1969) Cyclophosphamide therapy in the nephrotic syndrome in childhood. Brit. Med. J. I, 666,
   Wijdeveld, P.G.A.B. (1971) Some clinical and therapeutic aspects of the nephrotic syndrome in the adult. Folia Med. Neerl. 14, 4.

5. Het is niet mogelijk om op basis van renale angiografie een onderscheid te maken tussen een acute rejectie en een recente acute tubulusnecrose in een niertransplantaat.

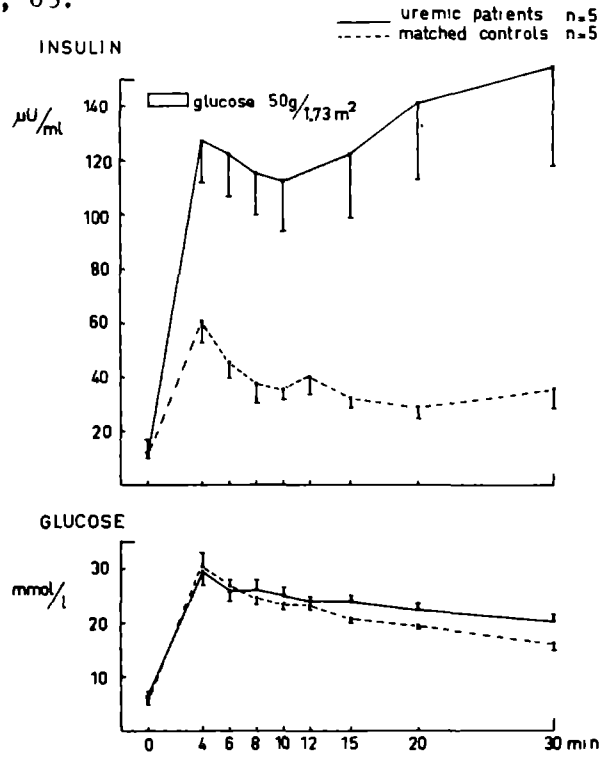
   Kaude, J., Slusher, D.H., Pfaff, W.W., Hackett, R.L. (1970) Angiographic diagnosis of rejection and tubular necrosis in human kidney allografts. Acta Radiol. Diagn. 10, 476.

6. De glucose infusietest volgens Cerasi en Luft geeft geen juiste indruk over de initiële insuline secretie. Deze bereikt haar hoogtepunt vóór het tijdstip, waarop in de test de eerste insulinebepaling wordt verricht.

Cerasi, E., Luft, R. (1963) Plasma insulin response to sustained hyperglycaemia induced by glucose infusions in human subjects. Lancet II, 1359.

7. In tegenstelling tot vele literatuurgegevens is bij lijders aan terminale nierinsufficientie na intraveneuze glucose-toediening niet alleen de late, maar ook de initiële insuline secretie sterk toegenomen. Hieruit blijkt dat de gestoorde koolhydraattolerantie bij deze patienten niet het gevolg is van een verminderde pancreasfunctie.

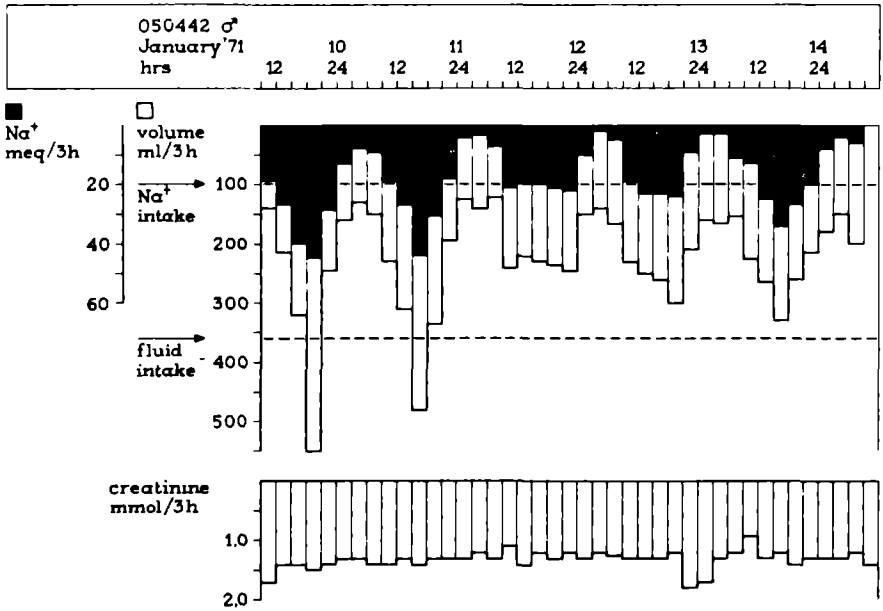
Horton, E.S., Johnson, C., Lebovitz, H.E. (1968) Carbohydrate metabolism in uremia. Ann. Int. Med. 68, 63.



Insuline- en glucosewaarden in het plasma na snelle intraveneuze toediening van glucose bij 5 patienten met uremie en bij 5 controlepersonen (Koene, R., van 't Laar, A., Benraad, Th., de Nobel, E., ongepubliceerde waarnemingen).

8. De opvatting van Berlyne e.a., dat tot 3 maanden na een niertransplantatie een omgekeerd dag- en nachtritme voor water en natrium bestaat, is niet algemeen geldig.

Berlyne, G.M., Mallick, N.P., Seedat, Y.K., Edwards, E.C., Harris, R., Orr, W.McN. (1968) Abnormal urinary rhythm after renal transplantation in man. Lancet II, 435.



Normaal dag- en nachtritme voor water en natrium bij een patient na niertransplantatie. De waarneming werd begonnen in de zesde week na de transplantatie (Wijdeveld, P., van Liebergen, F., Koene, R., ongepubliceerde waarneming).

9. Het onderzoeken en behandelen van grote aantallen patienten vormt op zich geen waarborg voor het verkrijgen van de vereiste vakkennis.

10. De aesculaap op motorvoertuigen wordt vrijwel uitsluitend gebruikt als statussymbool.